Xanthine oxidoreductase: A role in cell signalling

Rouquette, Magali

Award date:
1998

Awarding institution:
University of Bath

XANTHINE OXIDOREDUCTASE:
A ROLE IN CELL SIGNALLING.

submitted by Magali Rouquette

for the degree of PhD

of the University of Bath

1998

M Rouquette

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

First I would like to thank my supervisors Drs Roger Harrison and Bill Whish for their help and advice through my PhD. Thanks as well to Dr Stevens and Professor Blake (Postgraduate Medicine, University of Bath) for providing funding for my fees. Thanks to everyone in the labs 1.28 and 1.45, especially Richard Bryant and Dr Mustapha Benboubetra for the antibodies and Dr Stevie Boy Sanders for human milk XOR. Thanks also to David Tosh for his help with the confocal microscope.
Many thanks to Joan Whish for helping me growing 'the babies' and also for being my Cornish mum and a special thanks to Bill for providing me with bottles of port during my PhD and for being The Main Man. I also have to mention Professor Robert Eisenthal for teaching me French expressions that I did not know, dancing and walking strangely in the lab and most of all for his great songs, in particular 'Let the ball roll'. I have to give a big thanks to Katy (dearest Scotie), Stéphanie, Françoise and Susie Sue for their friendship. Stéphanie, depuis le temps que nous nous suivons j`espère que nous serons toujours dans le même pays! Finally, I have to mention the '19 Charlotte Street gang' for its so friendly atmosphere (most of the time!) and all the jokes and laughs. Rendez-vous dans 20 ans! And of course, a very special thanks to Andrew for his corrections and for finding the right word, not very surprising for Mr Big Word.
Enfin, en gardant le meilleur pour la fin, un très très grand merci à toute ma famille pour leur soutien et leur encouragements tout au long de ma thèse.

Cette thèse est dédiée à mes parents.

ABSTRACT

Cultured EA.hy 926 cells were used as a model system in which to characterise the occurrence and role of the enzyme xanthine oxidoreductase (XOR) in human endothelial cells. XOR can act as a source of hydrogen peroxide and superoxide, reactive oxygen species (ROS) that are increasingly seen as agents of signal transduction. EA.hy 926 cells were shown to express low levels of XOR enzymic activity that followed a characteristic pattern.

In view of the possible intermediary role of XOR-derived ROS in cytokine-stimulated expression of cell adhesion molecules on endothelial cells, effects of inflammatory cytokines on XOR activity in the endothelial cells were examined. However, no such effects were observed. The subcellular distribution of XOR on EA.hy 926 cells was investigated by confocal microscopy. As generally assumed, the enzyme was found in the cytoplasm, where, interestingly, it was particularly concentrated in the perinuclear region. XOR was also found to be located on the cell surface. Such an extracellular localisation in endothelial cells has important implications in the context of the inflammatory response. Intriguingly, the surface distribution of XOR was highly localised towards neighbouring cells, suggesting some role for the enzyme in cell-cell interactions.

The possibility that cell-derived extracellular agents (e.g. ROS) might influence XOR expression and cell behaviour was studied. Frequent changes of medium were found to prevent expression of XOR, as did catalase. Although these findings suggest the involvement of hydrogen peroxide, attempts to modify XOR expression or behaviour of the cells were unsuccessful using either hydrogen peroxide or SOD.

The mouse fibroblast cell line, L929, is also interesting in that, although it contains XOR mRNA, it only expresses enzyme activity after exposure to molybdenum, an essential cofactor of the enzyme. XOR protein was detected in these cells, both with and without exposure to molybdenum salts. The enzyme showed a similar distribution in all respects to that on EA.hy 926 cells. NADH oxidase activity, an intrinsic property of XOR, was shown to be present on the surfaces of EA.hy 926 and L929 cells.

LIST OF ABBREVIATIONS

Ab	Antibody
ARDS	Acute respiratory distress syndrome
BAECs	Bovine aorta endothelial cells
BBXO	Biozyme bovine xanthine oxidase
BPMVE	Bovine pulmonary microvascular endothelial cell
BRLE	Buffalo rat liver epithelial cell
Ca^{2+}	Calcium
CAM	Cell adhesion molecule
DHR	Dihydrorhodamine
DIC	Differential interference contrast
CNBr	Cyanogen bromide
DMSO	Dimethylsulphoxide
DPI	Diphenyleneiodonium chloride
EA.hy 926	Human endothelial cell line
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FCS	Foetal calf serum
Fe-S	Iron sulphur group
FITC	Fluorescein isothiocyanate
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HAECs	Human aortic endothelial cells
HB4a	Human mammary epithelial cell line

H_2O_2	Hydrogen peroxide
HPLC	High pressure liquid chromatography
H/R	Hypoxia/Reoxygenation
HRP	Horseradich peroxidase
HUVEC	Human umbilical vein endothelial cell
HXOR	Human xanthine oxidoreductase
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
I/R	Ischaemia/reperfusion
IU	International Units of activity
kDa	KiloDalton
L929	Mouse fibroblast cell line
LDL	Low density lipoprotein
LMVC	Lung microvascular cell
MEM	Minimum essential medium
Mo	Molybdenum
NA	Numerical aperature
NAD^+	Nicotinamide adenine dinucleotide
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
ND	Non detectable
NF-κB	Nuclear factor-Kappa B
NGS	Normal goat serum
NO	Nitric oxide
$O_2^{-}\cdot$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
PAEC	Pulmonary artery endothelial cell
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PFA	Formaldehyde
PMN	Polymorphonuclear leucocyte
PMSF	Phenylmethylsulphonyl fluoride

R123	Rhodamine 123
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polycrylamide gel electrophoresis
SEM	Standard error of mean
SOD	Superoxide dismutase
TGN	*Trans* Golgi network
Temed	N,N,N',N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
vWF	von Willebrand factor
XDH	Xanthine dehydrogenase-Type D
XO	Xanthine oxidase-Type O
XOR	Xanthine oxidoreductase

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CHAPTER 1
GENERAL INTRODUCTION

1.1. General enzymology of XOR

The molybdoenzyme, xanthine oxidoreductase (XOR), first purified from bovine milk, has been thoroughly studied (Bray, 1975, 1988; Massey and Harris, 1997). The enzyme has a wide specificity for reducing substrates and it is best known for its role in purine catabolism, catalysing the hydroxylation of hypoxanthine and xanthine to xanthine and uric acid respectively (Fig.1.1). These reactions were first described by Spitzer (1899).

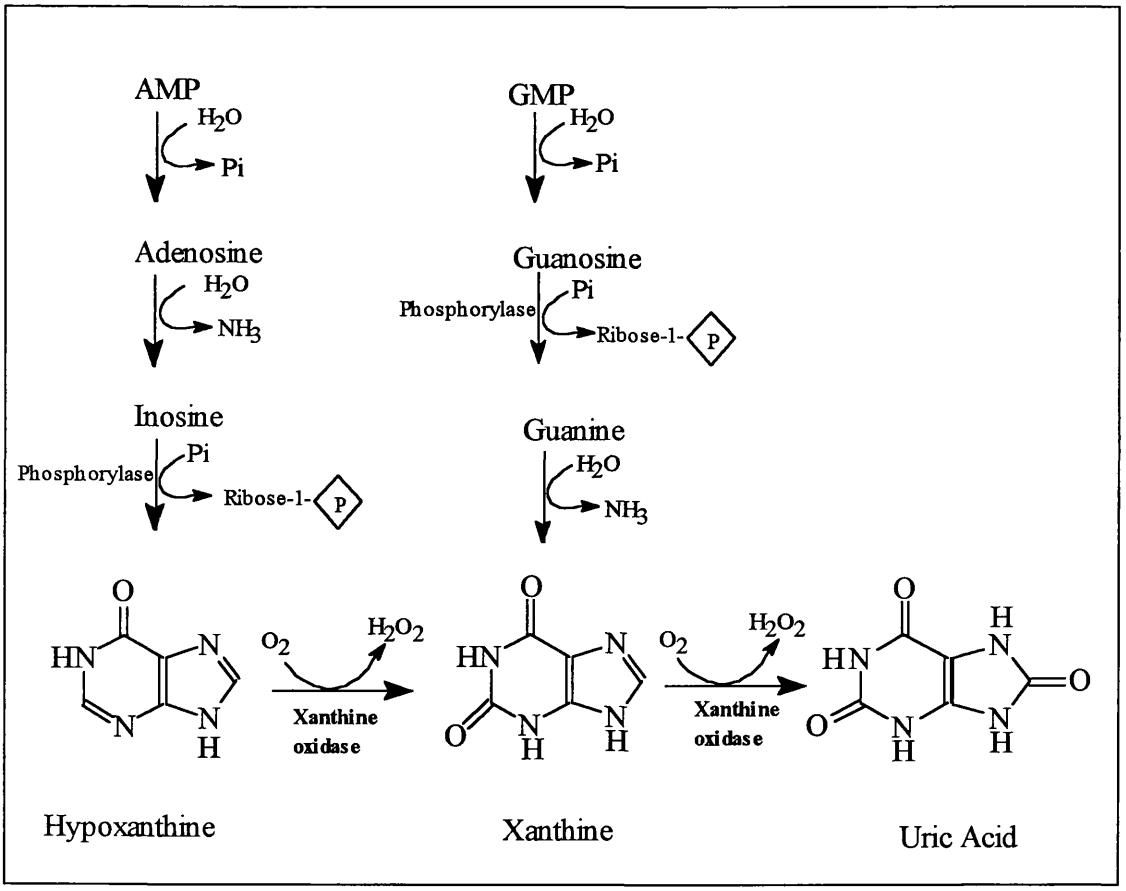


Fig.1.1: Catabolism of purine nucleotides to uric acid.
(Mathews and van Holde, 1990).

1.1.1. Electron transfer

XOR is a complex homodimer with a molecular weight of approximately 300 kDa. Each subunit contains one molecule of flavin adenine dinucleotide [FAD], one atom of molybdenum [Mo], and two iron sulphur [Fe-S] groups (Massey *et al*, 1969; Hart *et al*, 1970; Rajagopalan and Johnson, 1992). Electrons are passed to the physiological electron acceptors via the FAD centre. *In vitro*, the enzyme can also pass electrons via the iron sulphur centres to artificial electron acceptors such as methylene blue, as shown in Fig.1.2.

The molybdenum centre is the site where xanthine and all reducing substrates are oxidised, apart from reduced nicotinamide adenine dinucleotide (NADH), which is oxidised at the FAD centre.

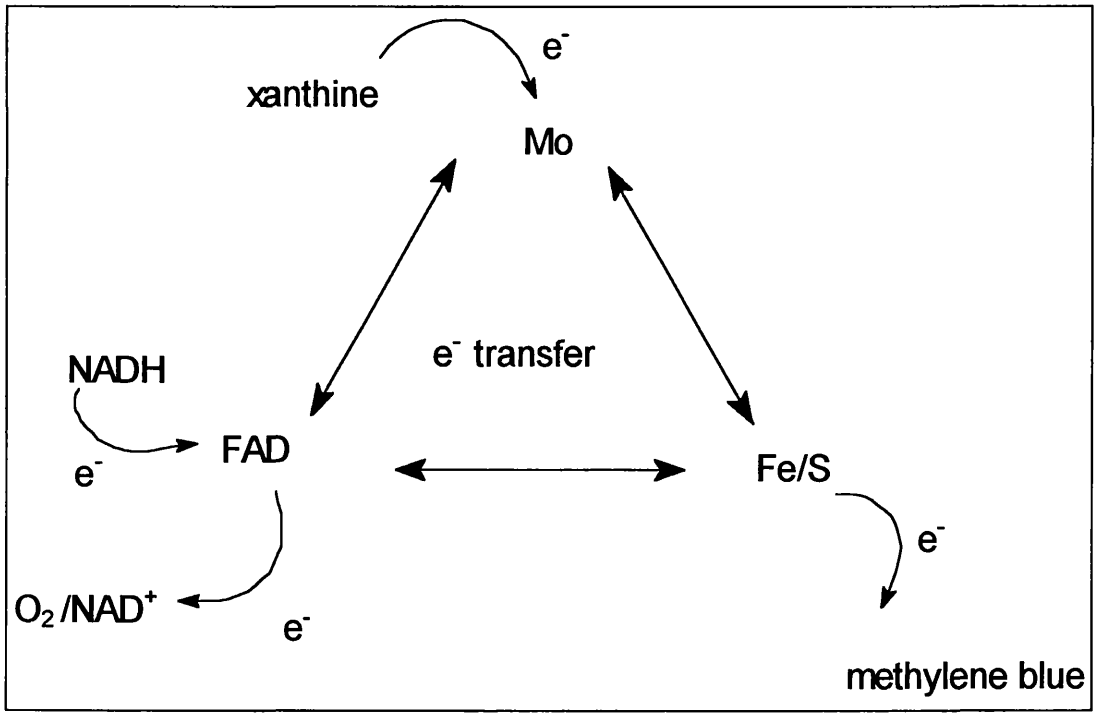


Fig.1.2: Electron transport for xanthine oxidase and sites of substrate interactions.

XOR can accept up to six electrons per subunit from the reducing substrate under normal conditions. These electrons can be transferred around the electron centres, with the iron sulphur centres probably acting as an electron sink to maintain the molybdenum in an oxidation state of six and the FAD as $FADH_2$ (Olson *et al*, 1974).

Equilibrium exists between the three redox centres. Each redox centre has its own affinity for electrons, independent of the number of electrons accepted from the reducing substrate and also independent of the redox state of the other centres. Transfer of electrons is fast between redox centres, 90 s^{-1} at 20°C (Hille and Anderson, 1991).

1.1.2. Inactive forms of the enzyme

As well as the active form, XOR purified from cows' milk also contains two inactive forms, desulpho and demolybdo. The desulpho form lacks an essential Mo=S grouping, containing Mo=O instead, and the demolybdo form lacks the molybdenum atom (Bray, 1975). These forms are, accordingly, inactive to most reducing substrates, which act at the Mo site. They will, however, still oxidise NADH which acts at the FAD site. A third inactive form, 'deflavo', lacking FAD, can be produced in the laboratory but does not occur *in vivo*. The inactive forms represent about 60% and 98% of XOR in bovine and human milk respectively (Abadeh *et al.*, 1992).

1.1.3. Xanthine oxidase and xanthine dehydrogenase interconversions

The enzyme exists in mammalian systems as Type D (dehydrogenase, XDH) and Type O (oxidase, XO) forms. XDH differs from XO in its ability to utilise NAD^+ as an electron acceptor during the oxidation of xanthine, whereas XO can only use molecular oxygen (Stirpe and Della Corte, 1969; Waud and Rajagopalan, 1976; Saito and Nishino, 1989). They are encoded by the same gene and can only readily be differentiated by their preference for electron accepting species (Xu *et al*, 1994 and 1995; Saksela and Raivo, 1996).

The dehydrogenase form predominates *in vivo*, but can be converted to the oxidase form, either irreversibly by proteolysis or reversibly by sulphydryl oxidation (Amaya *et al*, 1990). XDH contains fourteen sulphydryls per subunit (Della Corte and Stirpe, 1972). These sulphydryls can be oxidised to disulphides by treatment with thiol agents, and are, at least in part, involved in XDH to XO conversion (Waud and Rajagopalan, 1976).

Another mechanism of conversion (Fig.1.3) is by proteolytic cleavage of a 20kDa fragment, which is not actually lost from the complex. However, the nick in the peptide backbone is sufficient to destabilise the NAD^+ binding at the FAD site (Nishino and Tamura, 1991). Reversible and irreversible modification of XDH to form XO (Fig.1.3) result in conformational changes particularly surrounding the FAD unit. This leads to changes in flavin reactivity and loss of the NAD^+-binding site, resulting in negligible reactivity between xanthine oxidase and NAD^+ (Hille and Nishino, 1995).

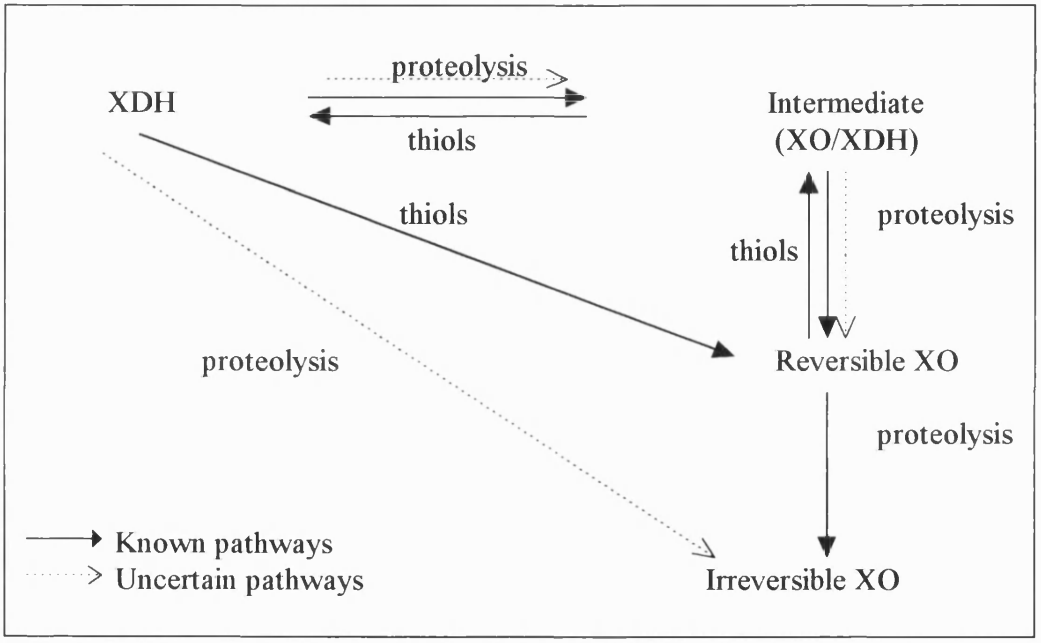


***Fig.1.3:* Pathways of interconversions between XDH and XO forms.**
(adapted from Parks and Granger, 1986).

1.1.4. Inhibition of XOR

There are two categories of inhibitors of XOR: those whose structures are analogues of purine substrates, and those that are not structurally related to the physiological substrates. The best known are structurally similar molecules including allopurinol (Fig.1.4) and oxypurinol (Moorhouse *et al*, 1987). Other less similar molecules include amflutizole (Werns *et al*, 1991) and BOF-4272 (Okamoto and Nishino, 1995). All these inhibitors inactivate the enzyme by blocking the Mo centre of XOR and do not affect NADH oxidation, which involves only the FAD centre (Sanders *et al*, 1997). Structurally very dissimilar inhibitors include compounds like cyanide, arsenite, formaldehyde and methanol (Parks and Granger, 1986).

Allopurinol acts as a substrate of XOR and undergoes oxidation to produce oxypurinol, which binds tightly to the reduced form of XOR. The oxypurinol-xanthine oxidase complex has a half-life of about 5h at 25°C (Spector *et al*, 1986).

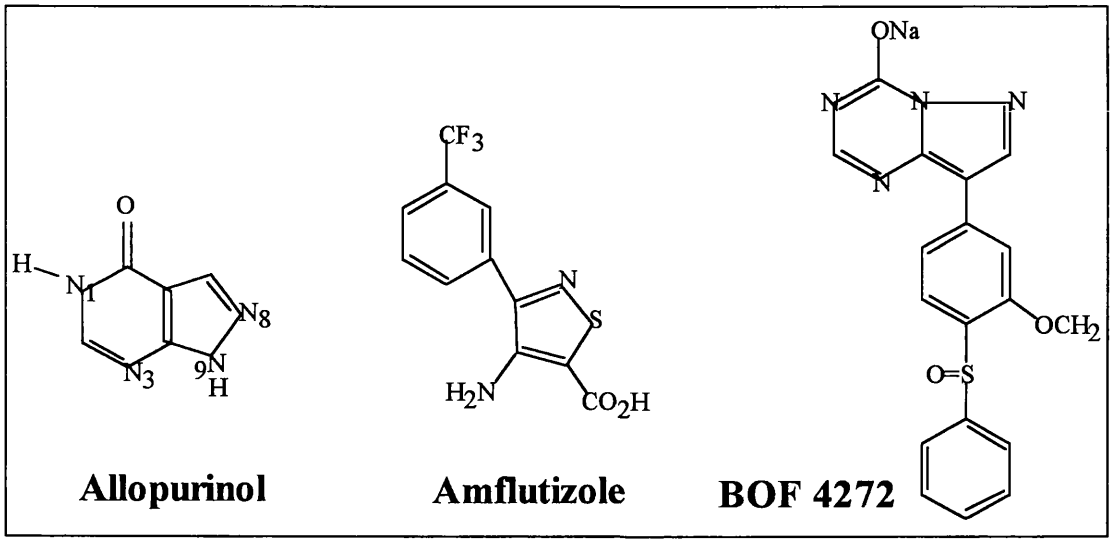


Fig.1.4: The chemical structures of XOR inhibitors.

1.1.5. cDNA sequences

cDNA for XOR for several species has been cloned and sequenced. The cDNA encodes a protein of approximately 1350 amino acids, which can be cleaved at two sites, generating the three distinct regions which make up the enzyme: the FeS domain (20KDa), the FAD domain (40KDa) and the Mo domain (85KDa) (Hille and Nishino, 1995). The full amino acid sequences have been determined from cDNA cloning of the enzymes from human liver (Ichida *et al*, 1993; Xu *et al*, 1994), rat liver (Amaya *et al*, 1990), mouse liver (Terao *et al*, 1992), bovine milk (Berglund *et al*, 1996), chicken liver (Sato *et al*, 1995) and from *Drosophila melanogaster* (Keith *et al*, 1987) and *Drosophila pseudoobscura* (Riley, 1989). The amino sequences are highly homologous (90% identity) among the rat, mouse, bovine and human enzymes. A weaker homology was observed between the mammalian and *Drosophila* enzymes (Fig.1.5).

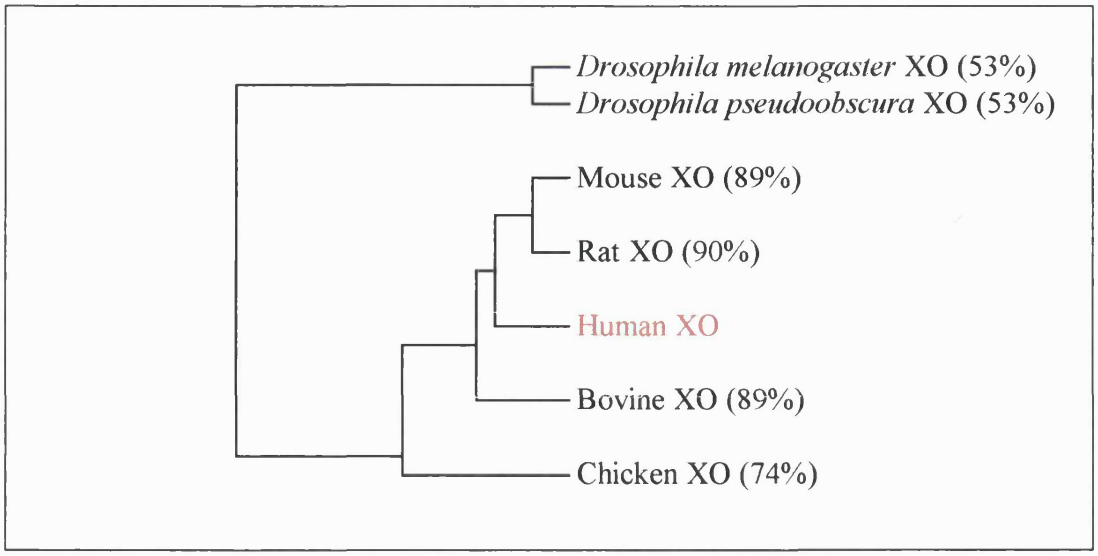


***Fig.1.5:* Percentage of homology of XO from different species compared to human XO.**

1.1.6. Human XOR

Human milk shows only low activity to xanthine and was, for many years, believed to contain little or no XOR (Carr *et al*, 1975). In 1987, Zeise and Zikakis were instrumental in showing the presence of XOR in breast milk, particularly colostrum, and reported an impure preparation of the enzyme that was highly contaminated by glycoprotein. Subsequently, apart from a brief report (Graham *et al*, 1989) of an enzyme preparation used for antibody production, the first characterisation of human milk XOR was that by Abadeh *et al* (1992). These workers showed that purified human milk XOR was essentially similar to that from bovine milk apart from very low specific activity to xanthine, which was attributed to a high proportion of inactive forms. The human milk enzyme has since been subjected to considerable further investigation which has demonstrated the presence of only very low (approximately 5%) levels of Mo, largely explaining its low conventional activity (Sanders *et al*, 1997; Godber *et al*, 1997). In contrast, the only other human XOR to have been purified, from post-mortem liver, has much higher activity to xanthine, equivalent to that of bovine milk enzyme (Krenitsky *et al*, 1986).

1.2. Origin and functions of reactive oxygen species (ROS)

ROS are constantly produced by metabolic reactions in the human body (Palmer and Paulson, 1997). Mitochondrial generation of O_2^{-}. represents the major intracellular source of oxygen radicals under physiological conditions. Other major sources of oxygen radicals are phagocytic cells such as neutrophils, monocytes and macrophages (Davies, 1995). Phagocyte-derived ROS are known to cause hepatic dysfunction with proteinases and may result in tissue damage (Spolarics, 1998). Superoxide production may also be brought about, when electrons leak from their carriers within the respiratory chain of mitochondria and pass directly onto oxygen (Fridovich, 1989; Imlay and Fridovich, 1991). Potentially major sources of ROS are thus mitochondrial leakage, phagocytic cells such as neutrophils, monocytes and macrophages, NADPH oxidase and XO.

XO, in passing electrons to molecular oxygen, generates the reactive oxygen species, superoxide anions and hydrogen peroxide (Bray, 1975). XDH can also react slowly with molecular oxygen to produce ROS. However, XO is generally considered to be the predominant form of XOR that is responsible for ROS production. Various stages in the reduction of molecular oxygen are shown in Fig.1.6. ROS include singlet oxygen (O_2), hydrogen peroxide (H_2O_2), the superoxide anion (O_2^{-}.) and the hydroxyl radical ($OH^{\cdot}$). The Fenton reaction corresponds to the oxidation of reduced iron (Fe^{2+}) by H_2O_2 to generate $OH^{\cdot}$.

ROS are thought to be important in an increasing number of physiological and pathological processes. They have a positive role in the defence against microorganisms and a negative role in damaging cell components (Khan and Wilson, 1995). ROS have also been implicated as second messengers. One of the most cited examples is the regulation pathway of the transcription factor, NF-κB. The inactive complex, NF-κB–IκB, is present in the cytoplasm of the cells, and is induced to dissociate by phosphorylation. NF-κB is then free to enter the nucleus and to induce genes involved in inflammation (Baeuerle and Baltimore, 1988 a/b; Beg *et al*, 1992; Brown *et al*, 1993; Henkel *et al*, 1993; Sun *et al*, 1993). ROS, particularly H_2O_2, have

been implicated in this process. In 1991, Schreck and coworkers reported the induction by H_2O_2 of the expression and replication of HIV-1 in a human T cell line, an effect mediated by NF-κB. Moreover, DNA binding of the AP-1 transcription factor has also been shown to be induced by H_2O_2 (Devary *et al*, 1991). Low amounts of non-toxic ROS were found to increase the transient concentration of Ca^{2+} in cultured human and rat endothelial cells, modulating receptor-mediated calcium signalling. However, high doses of ROS caused the concentration of Ca^{2+} to increase via extracellular sources, leading to the process of cell death (Volk *et al*, 1997). Pathogenic roles of ROS have been implicated in ischaemia-reperfusion injury (Granger *et al*, 1981; Sussman and Bulkley, 1990), in atherosclerosis (Ohara, 1993), and in inflammatory and autoimmune rheumatic disease (Miesel and Zuber, 1993).

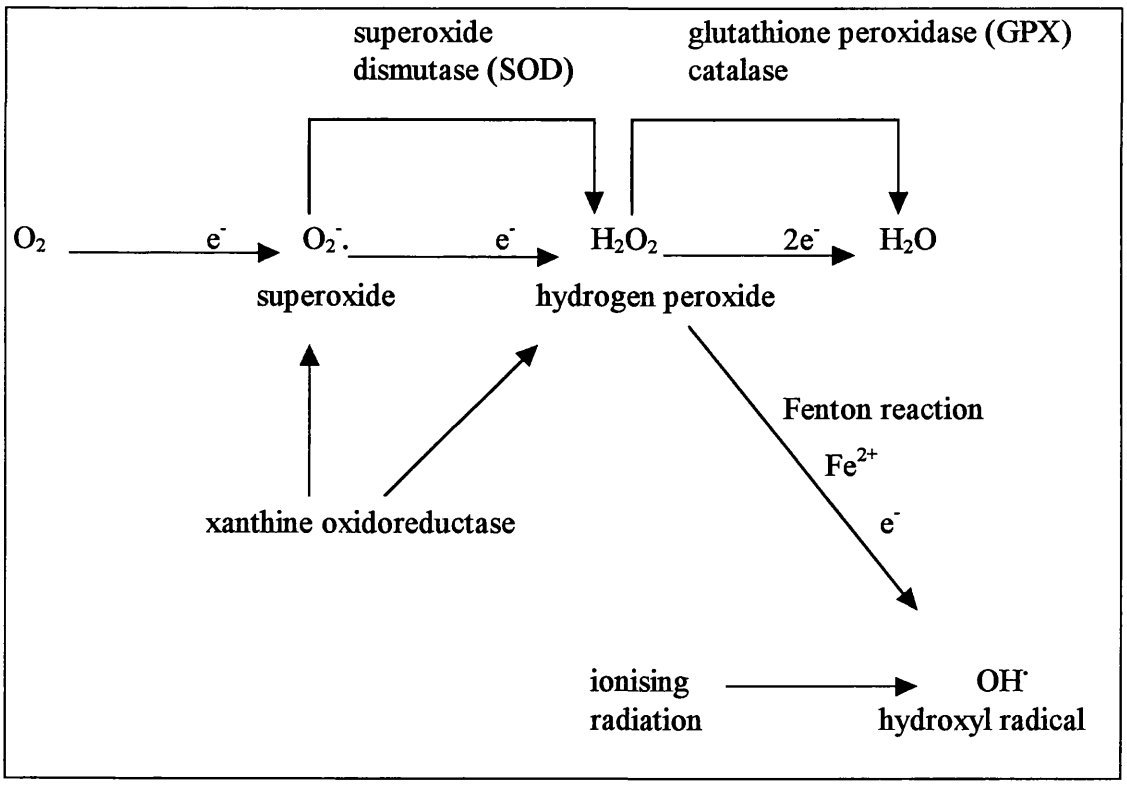


Fig.1.6: ***The reduction of molecular oxygen to water and partial reductions yielding to ROS.***
(Adapted from Vanden Hoek et al, 1997).

1.3. Role of XOR in ischaemia/reperfusion (I/R)

ROS were proposed by Granger *et al* (1981) as central mediators of the cell injury that occurs in post-ischaemia reperfusion. The proposed mechanism involves the following sequence of events. During ischaemia, energy depletion initiates the catabolism of purines and the accumulation of hypoxanthine. Concurrently, loss of energy status leads to an influx of Ca^{2+} into the cytosol and the activation of proteases. These proteases convert the native dehydrogenase form of XOR to XO. On reperfusion, O_2 is readmitted to the tissues, now containing high concentrations of hypoxanthine and XO, which combine to yield destructive ROS (Fig.1.7).

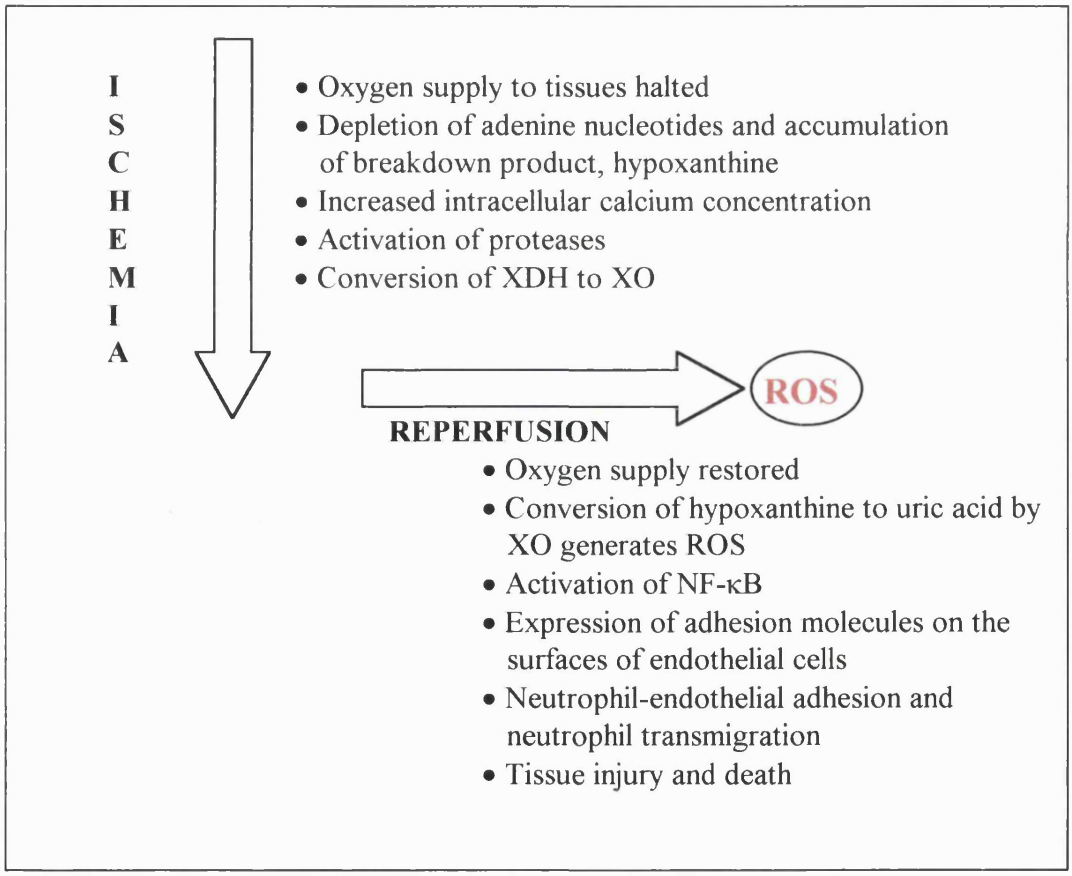


Fig.1.7: Biochemical changes which occur during and after ischaemia. *(Adapted from Granger et al, 1981).*

This hypothesis has been extensively examined by subsequent authors and in many cases XOR has been implicated in the initial burst of ROS in post-ischaemic reperfusion (Zweier *et al*, 1988; Michiels *et al*, 1992).

However, several aspects of the original scheme have been questioned, particularly the extent and time scale of XDH to XO conversion (Kooij *et al*, 1994; Frederiks and Bosch, 1996). Fig.1.8 represents a modified scheme involving endothelial XOR and also neutrophils during ischaemia-reperfusion. During ischaemia, XDH is converted to XO within the endothelial cells. When reperfusion occurs, XO catalyses superoxide generation from molecular oxygen and the resulting production of ROS induces cell injury. Neutrophils can be attracted to the area either by the cell injury or by the chemotactic properties of superoxide. They are then activated, so amplifying the system and inducing further injury to endothelial and parenchymal cells. The initial burst of ROS which stems from the vascular endothelium is hypothesised to trigger the adhesion and migration of leucocytes across the endothelium and into the surrounding tissue (Bulkley, 1994; Brass, 1995). A fundamental assumption in these hypotheses is that xanthine dehydrogenase (80% predominant) is converted to xanthine oxidase during ischaemia. However, it has been shown that the conversion from XDH to XO by proteolysis is both slow and involves only a limited proportion of the enzyme (Nishino and Tamura, 1991). Therefore, the situation is likely to be more complex than originally thought. Alleviation of ischaemia-reperfusion injury by allopurinol has often been quoted in support of Granger's hypothesis (Martz *et al*, 1989; Palmer *et al*, 1990), although its relevance has been questioned both on the grounds of the substrate activity of allopurinol (Massey *et al*, 1970) and its ability to scavenge ROS (Bulkley, 1994).

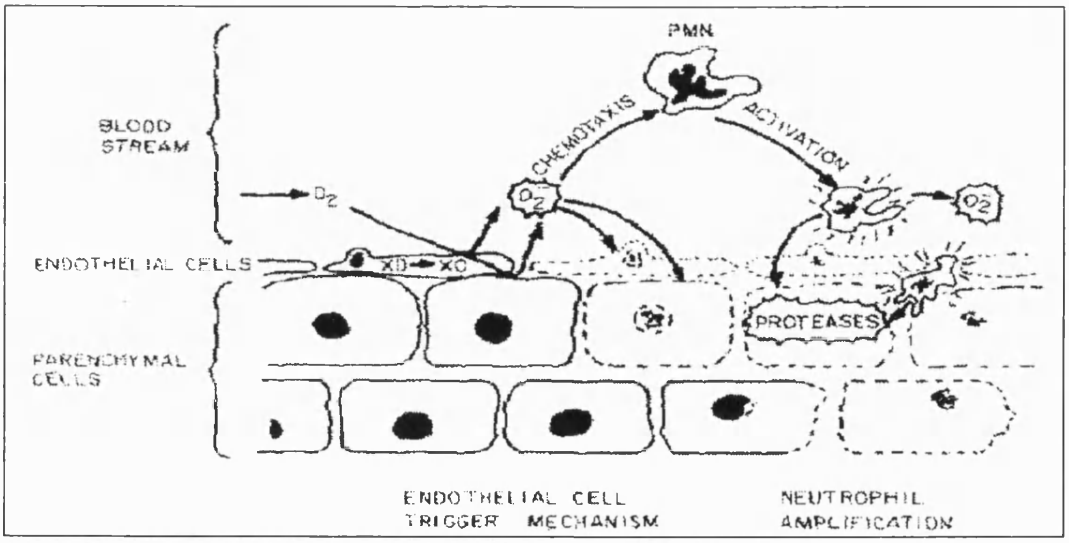


***Fig.1.8:* Endothelial cell trigger hypothetical mechanism:**
PMN: polymorphonuclear leucocyte (Ratych et al, 1987).

1.4. Inflammation and cytokines

Ischaemia and inflammation are closely linked. As shown in the scheme of Fig. 1.8, ROS produced after human endothelial cells have been subjected to hypoxia/reperfusion have been implicated in increasing neutrophil adherence (Palluy *et al,* 1992), a key stage in inflammation. Vascular endothelial cells play an important role in controlling the migration of leucocytes to sites of inflammation and regulating the development of immune and inflammatory events in the tissue (Springer, 1995; Suzuki *et al,* 1991; Varani and Ward, 1994). Migration of leucocytes toward the inflamed tissue is modulated by the release of a number of inflammatory mediators which include cytokines, bacterial endotoxins and degenerative products of the inflamed tissues. During the inflammation process, endothelial cells are induced to undergo major changes in gene regulation and surface expression of important cell adhesion molecules (CAMs) (Baumann and Gauldie, 1994), and ROS have been implicated in these processes (Varani and Ward, 1994). CAMs interact specifically with neutrophils and other circulating leucocytes to slow their rate, initiate *trans*-endothelial passage and extravasation, and allow subsequent migration into the tissue (Lasky, 1992). While patterns of expression of CAMs are well defined, mechanisms controlling their induction are poorly understood.

1.5. Natural antioxidant defences in cells

Excess levels of ROS are controlled by specific antioxidant mechanisms in healthy persons. However, a pathological condition known as oxidative stress can result from an increase in ROS production or a decrease in antioxidant defences (Grisham, 1994; Halliwell, 1994; Neubauer and James, 1994). Enzymes specialised in the elimination of ROS include catalase, superoxide dismutase (SOD), and the glutathione peroxidase system (GSH) (Schreck and Baeuerle, 1991).

1.5.1. Catalase

This iron-containing enzyme catalyses the conversion of hydrogen peroxide to water and molecular oxygen. The reaction is effectively a dismutation, involving oxidation of one molecule of H_2O_2 and the reduction of the other (Fig.1.9).

1.5.2. Superoxide dismutase

The superoxide dismutases are a family of metalloenzymes that also catalyse dismutation, in this case of superoxide (Fig.1.9). All members of the SOD family utilise a transition metal at their active site. In eukaryotes, a cytosolic form of the enzyme, Cu/Zn-SOD, contains copper and zinc. A manganese containing form, Mn-SOD, is present in mitochondria and bacterial cells. Finally, an extracellular form of SOD is attached to the endothelium by heparin-binding and has been implicated in protection from endothelium-derived oxidative stress (Mathews and van Holde, 1990; Davies, 1995). Mitochondrial SOD is highly inducible by cytokines (Wong, 1995).

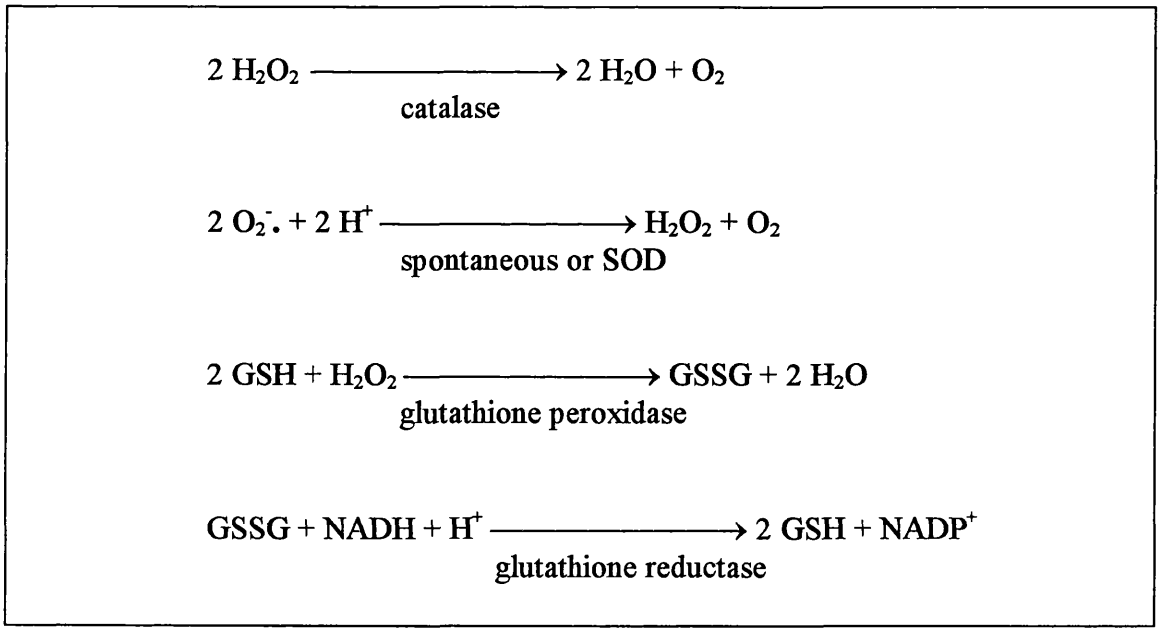


Fig.1.9: Reactions catalysed by catalase, SOD and glutathione peroxidase.
GSH: reduced glutathione; GSSG: oxidised glutathione (Mathews and van Holde, 1990).

1.5.3. Glutathione peroxidase and glutathione reductase

Glutathione peroxidase reduces H_2O_2 to water, simultaneously oxidising glutathione. Two molecules of GSH are oxidised to form the disulphide compound GSSG during the reduction of H_2O_2. Glutathione reductase utilises NADPH to re-reduce one molecule of GSSG to two molecules of GSH, thus permitting the continuous action of glutathione peroxidase (Fig.1.9).

1.6. Distribution of XOR

XOR has been detected in all species studied, from bacteria to man. In most human tissues, the levels of XOR activity have been reported to be low (Parks and Granger, 1986). XOR has been purified from human milk and shown to have very low activity to xanthine. Preliminary purification of XOR from human heart indicates this enzyme to be similar (Abadeh *et al*, 1992). Liver and intestine, on the other hand, show relatively high xanthine oxidoreductase activity (Parks and Granger, 1986; Sarnesto *et al*; 1996), and the enzyme purified from liver showed high specific activity very similar to those of the bovine milk and rat liver enzymes (Krenitsky *et al*, 1986). It is possible but not proven that XOR in human tissues can be classified in two groups. On the one hand, 'high level activity' enzyme occurs in a limited number of tissues such as liver and intestine; and on the other hand, 'low level activity' enzyme is present in breast milk, heart and probably most other tissues. Study of purified human milk XOR indicates that high concentrations of the inactive XOR are present *in vivo*, suggesting the possibility of post-translational regulation of activity in response to certain stimuli. 'Desulpho'-'sulpho' enzyme conversion of bovine milk XOR has been effected by incubation of the reduced enzyme with sulphide ion, resulting in the activation of the enzyme (Wahl and Rajagopalan, 1982), and although the mechanism is unknown, evidence of post-translational upregulation of XOR in the human epithelial cell line HB4a has been reported by my colleagues (Page *et al*, 1998). A number of cytokines have been shown to stimulate XOR activity in these cells without a corresponding increase in specific mRNA or XOR protein. Endothelial cells appear to belong to the 'low level activity' group of human XOR enzymes.

The first report mentioning the presence of XOR in endothelial cells was from Jarasch and coworkers in 1981. Since this date, numerous studies have involved endothelial cells and tissues from bovine, porcine, rat and human sources (Appendix I). XOR has been mainly detected in capillary endothelial cells (Bruder et al, 1983; Jarasch *et al*, 1986; Samra *et al*, 1991 etc.). Detection techniques include histochemistry (Kooij *et al*, 1992; Frederiks *et al*, 1993a, 1993b; Hellsten-Westing, 1993; Moriwaki *et al*, 1993), fluorimetric assays (Bhat *et al*, 1992; Dupont *et al*, 1992; Michiels *et al*, 1992; Panus *et al*, 1992; Hassoun *et al*, 1994; er-Martinez *et al*, 1994; Poss *et al*, 1996; Zulueta *et al*, 1997), spectrophotometric assay of uric acid formation (Ratych *et al*, 1987; Palluy *et al*, 1992; Wiezoreck *et al*, 1994; Zweier *et al*, 1994), HPLC (Rodell *et al*, 1987; Phan *et al*, 1989; Terada *et al*, 1991, 1992, 1993; Wakabayashi *et al*, 1995), radioassays (Jarasch *et al*, 1986), chemiluminescence (Hellsten *et al*, 1997), and spectrofluorimetric measure of the release of H_2O_2 (Yang and Block, 1995).

1.7. The role of endothelial cells

Endothelial cells are one of the most widely distributed cell types. They form a continuous layer, lining the blood vessels of the entire vascular system. Vascular endothelial cells interact with their environment at three different interfaces (Fig.1.10):

* The basal surface is the endothelial attachment to the basement membrane of the underlying vessel intima. The membrane is composed of plasma proteins (such as heparin), collagens; laminin; glycoprotein and fibronectin.
* The lateral surfaces interact with adjacent endothelial cells to form a junction which regulates permeability and maintains the cell surface polarity.
* The apical or luminal surface interacts with the blood and its cellular elements. The apical surface contains adhesion molecules that can bind leucocytes or platelets (ICAM-1, ICAM-2 etc.) and it plays a vital role in maintenance of homeostasis and vascular events. It is coated with peripheral components such as plasma proteins (heparin), albumin and fibrin, glycoproteins etc. (Bradley *et al*, 1995; De Angelis *et al*, 1996).

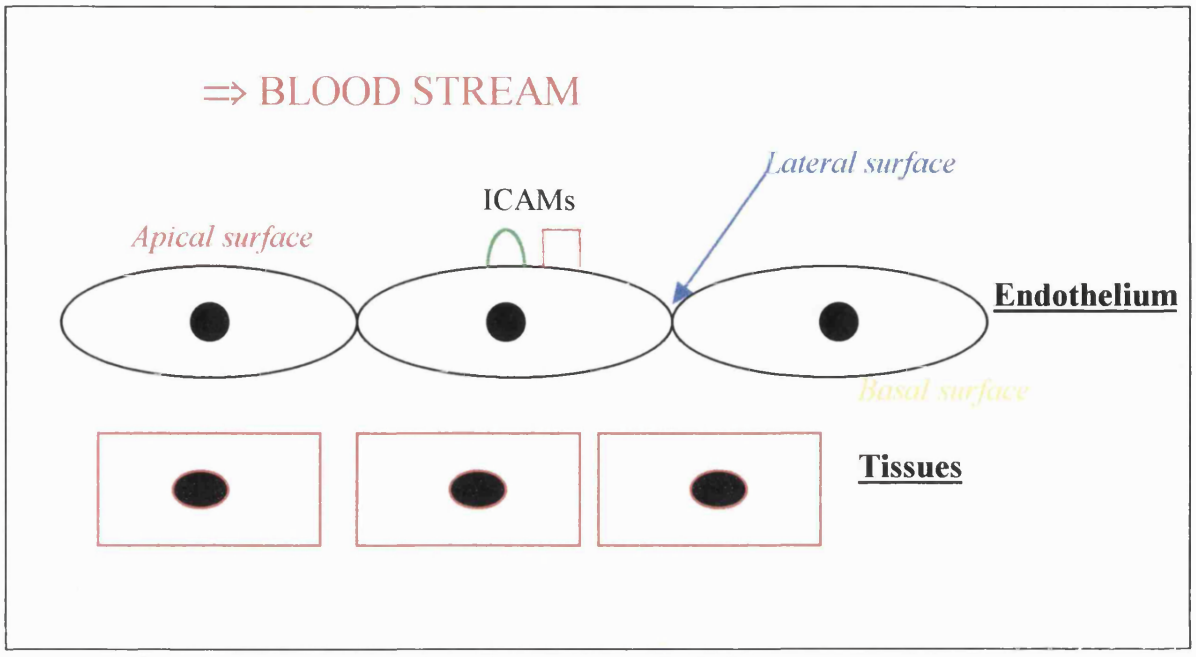


Fig.1.10: ***Schematic representation of vascular endothelium.***

The endothelium has a variety of roles, the most obvious being the exchange of metabolites, nutrients or secretory products between the blood and interstitial fluids. The endothelial cells also regulate membrane transport, coagulation and fibrinolysis, synthesis and release of vasoactive hormones, etc. Vascular smooth muscle cells as well as endothelial cells are capable of producing reactive oxygen species from a variety of enzymic sources (Harrison, 1997a).

1.8. Communication between cells

1.8.1. Cell Signalling

Endothelial cells, like other cells, need to communicate with each other or with other cells in order to coordinate their movement, metabolic activity and growth. There are three general mechanisms for intercellular signalling in multicellular organisms. The first is direct contact between the cells, which can communicate via cytoplasmic bridges. Cytoplasmic bridges allow intercellular signal molecules to pass from one cell to the other, without the necessity of secretion into the extracellular fluid. In animal cells, these bridges are referred as gap junctions. Cells can also communicate via

extracellular signal molecules, also called first messengers. First messagers are usually secreted by one cell to activate or cause a response in another cell. Finally, surface molecules on one cell can interact directly with receptor molecules on another cell (Fig.1.11). This last mechanism is not fully substantiated (Hardie, 1991).

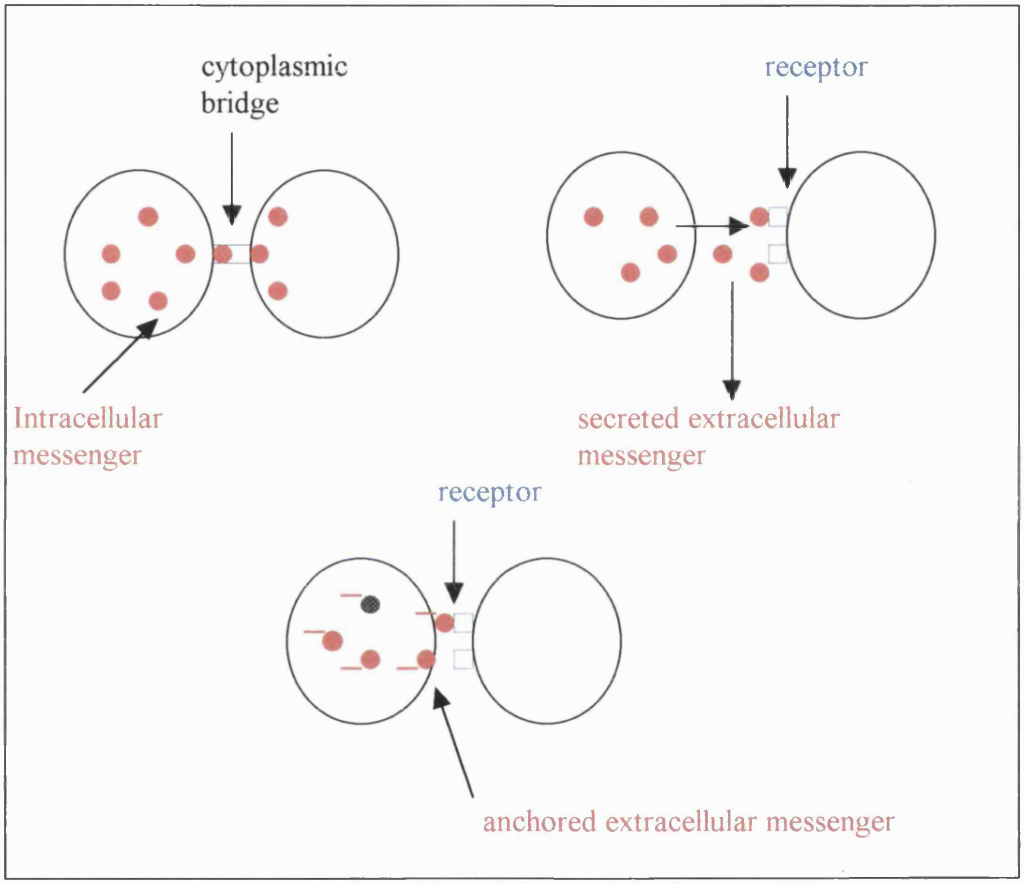


***Fig.1.11:* Possible mechanisms of cell signalling (Hardie, 1991).**

1.8.2. Secretion pathway

Most first messagers are released from cells via exocytosis, a phenomenon which includes the fusion of secretory vesicles with the plasma membrane. Proteins that enter the endoplasmic reticulum (ER) are converted to glycoproteins by the covalent attachment of oligosaccharide side chains. At a number of locations, the outer membrane of the nuclear envelope is continuous with the endoplasmic reticulum. The proteins are then transported from the ER to the Golgi apparatus by means of transport vesicles. The Golgi consists of a collection of flattened, membrane

bounded sacs, which are like stacks of plates (Fig.1.12). Each Golgi stack has two distinct faces; *cis*, the entry face and *trans,* the exit face. The Golgi apparatus modifies the proteins and dispatches them. Those destined to be secreted are transported to the plasma membrane in transport vesicles that fuse with the membrane (Hardie, 1991; Barritt, 1992 and Alberts *et al*, 1998).

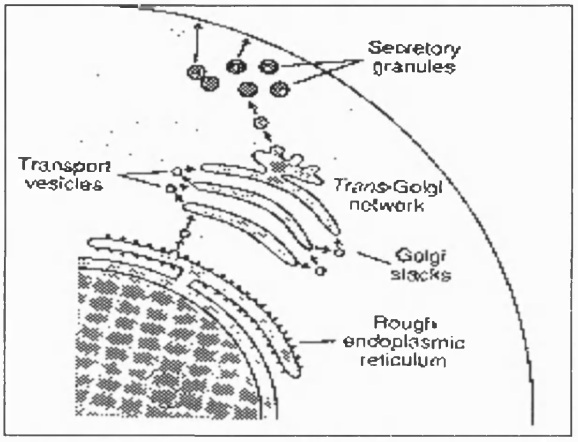


Fig.1.12: The pathway of secreted proteins (Hardie, 1991).

1.8.3. Secretion of XOR

The intracellular location of XOR is not clear. It is associated with the milk fat globule membrane in milk, but can also be detected as a soluble form (Briley and Eisenthal, 1974). In other tissues, it is usually reported to be cytosolic. Jarasch *et al* (1981), used both light and electron microscopic immunohistochemical procedures to show that XOR is located throughout the cytoplasm of bovine capillary endothelial cells. Work on rat hepatocytes concluded that the enzyme was exclusively cytosolic with no significant association with intracellular organelles (Ichikawa *et al*, 1992). Moriwaki and coworkers (1993) showed the presence of XOR in human liver cytosol. The presence of XOR on the outer cell surface has also been briefly reported in bovine aortic endothelial cells (Bulkley, 1991; Schiller *et al*, 1991) and more recently by Vickers *et al* (1998).

1.9. Aims:

The aims of this work were to use cultured human endothelial cells, EA.hy 926, as a model system in which to study XOR. Initially, activation of XOR via cytokines was investigated in view of a possible intermediary role of XOR-derived ROS in cytokine-stimulated expression of CAMs on endothelial cells. In the course of these studies, interesting patterns of XOR expression were observed in the EA.hy 926 cells. These observations led to demonstration of the extracellular localisation of the enzyme and to exploration of its role in intracellular signalling.

CHAPTER 2

GENERAL MATERIALS AND METHODS.

All the chemicals used were ordered from Sigma (Poole, Dorset), BDH (Poole, Dorset), or Fisons (Loughborough, Leicestershire), unless otherwise stated.

2.1. Cell culture

2.1.1. Materials and instruments

Sterile foetal calf serum (FCS) was obtained from Globepharm (Esher, Surrey). Sterile plastic flasks for cell culture (25, 75, and 150 cm^2) as well as disposable sterile cryogenic vials (2ml) were ordered from Western Laboratories Services (Hampshire). Phosphate buffered saline (PBS) tablets and trypan blue (0.4% solution) were from ICN Pharmaceuticals (Oxon). Needles and syringes were provided by South Syringe Services (Bristol). Interferon-γ (IFN-γ) was from Calbiochem-Novabiochem (Nottingham). L929 cells, glutamine, minimal essential medium (MEM), and M199 were obtained from ICN Flow (High Wycombe). Haemocytometers were purchased from Gallenkamp-Weber Scientific (Lancing, Surrey). Centrifuges used were MSE Micro Centaur, Beckman Instruments L5-50B (for 175,000g) and a Beckman Instruments TL 100 (for 500,000g). Cell culture was carried out in a Microflow culture hood and a MSE 150 Watt Ultrasonic Disintegrator Mk2 was used for sonication.

2.1.2. Cell lines

Essentially, the same procedures of maintenance and subculture of the cells were used for the different cell lines.

2.1.2.1. EA.hy 926

EA.hy 926, a permanent endothelial cell line, was a gift from Dr A. George, Hammersmith Hospital (London). The cells were maintained in RPMI 1640 medium, with 10%(v/v) FCS, penicillin/streptomycin [5000 IU/ml for penicillin and 5mg/ml in 0.9%(v/v) sodium chloride for streptomycin]. They show typical endothelial cell characteristics and were positive for von Willebrand factor (vWF) by immunofluorescence (Fig.3.1).

2.1.2.2. L929

L929 is a mouse fibroblast cell line established in1943 by W.R. Earle. It was one of the first cell lines to be established in continuous culture. The cell line was derived from the subcutaneous and adipose tissue of a 100 day old male C3H/An mouse. The cells were maintained in MEM with 10%(v/v) FCS, penicillin/streptomycin [5000 IU/ml for penicillin and 5mg/ml in 0.9%(v/v) sodium chloride for streptomycin] and 2mM glutamine. The pH of the medium was adjusted to pH 7.4 with NaOH.

2.1.2.3. Human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained from human umbilical veins, kindly donated by the nursing staff of the Princess Anne Wing, Royal United Hospitals (Bath). Primary cultures were isolated using a method modified from Jaffe *et al* (1973). HUVECs were cultured in M199 with 2mM L-glutamine, supplemented with 20%(v/v) FCS, heparin (17 IU/ml), penicillin (100 IU/ml), streptomycin (100μg/ml) and endothelial cell growth factor (30μg/ml).

2.1.3. Cell culture maintenance and subculturing

Attached cells were initially washed twice with prewarmed PBS (5ml/wash). Trypsin [1ml; 0.5mg/ml] diluted with EDTA/PBS (3ml) was added to detach the cells. The trypsinisation reaction was stopped by the addition of an equal volume of prewarmed medium (4ml for a 75 cm^2 flask). The cells were removed by trituration using a syringe and a No19 needle to avoid clumps of cells, and then counted in a haemocytometer, using a 1:1 dilution with 0.04%(w/v) trypan blue in order to check

the viability of the cells. The cells were then seeded at the required density, in a 75 cm^2 plastic tissue culture flask, with prewarmed medium (30ml), and incubated at 37°C, humidified with 5% CO_2/95% air(v/v). The medium was then changed every three to four days, until cells formed a confluent monolayer. All experiments were conducted on cells with less than thirty passages after thawing from liquid nitrogen. It should be noted that volumes quoted were for a 75 cm^2 plastic tissue culture flask; for a 25 cm^2 flask, the volumes were divided by two.

2.1.4. Preparation of cell supernatant for XOR assay

Cells were trypsinised using the above procedure, transferred into centrifuge tubes and centrifuged at room temperature for 5min at 1500 rpm (100g). The supernatant was discarded and the pellet was resuspended in Cell Buffer [1.2ml; 50mM phosphate buffer, pH 7.4, containing 0.1mM EDTA, 0.1mM phenylmethylsulphonyl fluoride (PMSF), pepstatin A (1μg/ml), leupeptin (1μg/ml), antipain (1μg/ml) and aprotein (1μg/ml)]. Stocks of these inhibitors were dissolved in distilled water except for pepstatin A which was dissolved in methanol. The cells were sonicated by using a 3mm probe for 20sec (power setting six). A benchtop microcentrifuge tube cooler was used during sonication to keep the temperature constant at 5°C. The resulting cell homogenates were ultracentrifuged at 4°C, either for 25min at 35,000 rpm (175,000g), or for 10min at 100,000 rpm (500,000g). These supernatants were then used for fluorimetric assay (Section 2.3.2), for Western blotting (Section 2.4.3), and when necessary, for XOR protein ELISA (Section 2.6).

2.1.5. Cryopreservation

The cells were trypsinised, as described above, and the trypsinisation was stopped by adding medium and 10% dimethylsulphoxide (DMSO). The cells were aliquoted in cryogenic tubes and stored for 24h at -70°C, before being immersed in liquid nitrogen. Aliquots were thawed by immersion in a 37°C waterbath and the cells were set in a 25 cm^2 flask with the appropriate medium. After 24h, the medium was changed to remove the toxic effects of DMSO. When the cells were confluent, they were trypsinised and transferred to 75 cm^2 flasks at the required density (Section 2.1.3).

2.2. Protein estimation

2.2.1. Materials

Bio-Rad protein dye reagent concentrate was obtained from Bio Rad (Hemel, Hempstead, Hertfordshire). Plastic cuvettes (1ml) were from Griffiths and Nielson Plastics Ltd (Billingshurst, Sussex). The 595nm absorbance was read on a Multimode computing UV spectrophotometer, CE6600 from CECIL Instruments Limited (Cambridge).

2.2.2. Method

The protein estimation was carried out on cell supernatants, using the method developed by Bradford (1976). Bovine serum albumin (BSA) in assay buffer (1mg/ml) was used as a standard, in a range from 2 to 10µg in a volume of 100µl. Each sample was appropriately diluted and a final volume of 100µl was used. The Bio-Rad protein assay dye reagent (1ml; dilution1:5) was added and the mixture was left for 15min to react. The absorbance at 595nm was read on the spectrophotometer.

2.3. Assays of XOR enzymic activity

2.3.1. Chemicals and instruments

The fluorimetric assay was carried out on a Perkin Elmer (Beaconsfield, Bucks) LS-5B luminescence spectrometer. The xanthine oxidoreductase inhibitors amflutizole (Werns *et al*,1991) and (-) BOF 4272 (Okamoto and Nishino, 1995) were gifts from Professor D.R. Blake (Postgraduate Medicine, University of Bath). Human milk xanthine oxidoreductase was prepared by Dr Steve Sanders (School of Biology and Biochemistry, University of Bath). Absorbances were read on a Multimode computing UV spectrophotometer, CE6600 from CECIL Instruments Limited (Cambridge). Acrylic cuvettes (1ml) were from Griffiths and Nielson Plastics Ltd (Billingshurst, Sussex). The cells (Section 2.1.2) were grown on four chambered polystyrene glass sides obtained from Nunc Inc. (Naperville, IL, USA).

2.3.2. Fluorimetric assay of pterin oxidation

The fluorimetric assay used was based on that described by Beckman *et al* (1989). This method was modified for our cell lines (principally EA.hy 926 cells) to detect XOR enzymic activity in cell homogenates (Rouquette *et al*, 1997). The fluorimeter was used with an excitation wavelength of 345nm, and the emission wavelength set at 390nm with 5nm band width slits. The recording was at scale five or one, at a chart speed of 0.5cm/min. All the reagents were first brought to room temperature. A baseline was obtained with a mixture of cell supernatant (0.5ml) and buffer (50mM potassium phosphate, pH 7.4, containing 0.1mM EDTA; 0.48ml). When a stable baseline was obtained, 1mM pterin (10µl) and 1mM methylene blue (10µl) were added, each to a final concentration of 10µM. The increase in fluorescence was recorded and this value was a measure of the total (xanthine oxidase and xanthine dehydrogenase) activity in the cell supernatant. The specificity of the activity was checked by adding 50µM of allopurinol (50µl), a specific competitive inhibitor of xanthine oxidoreductase. Finally, the reaction was calibrated by addition of a range of concentrations of 10µM isoxanthopterin (2 to 4µl, repeating addition five times), see Fig.3.4.

From the isoxanthopterin standard curve, a linear regression best fitted line was plotted. Rates from the chart recorder, expressed in fluorescence units/min (F/min), were converted to pmoles isoxanthopterin/min by plotting this rate into the linear regression line. The value was converted to pmoles isoxanthopterin/min/ml by dividing the results obtained by the volume of the sample (in most of the case 0.5ml). Finally, the result was expressed in pmoles/min/mg protein.

2.3.3. Determination of xanthine and dehydrogenase activities of XOR

The oxidase content of XOR was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295nm, using an absorption coefficient of $9.6mM^{-1}cm^{-1}$ (Avis *et al* 1956). Assays were performed at 37°C, in a 1ml cuvette in 50mM sodium/Biocine buffer, pH 8.3 (800µl) containing 100µM xanthine (100µl). The sum of oxidase and dehydrogenase contents was determined as above but in the presence of 0.5mM NAD (100µl).

The effects of a number of inhibitors of XOR were investigated. In each case, a linear rate was established for 2-3min before addition of the inhibitor. These were prepared as follows. Allopurinol was dissolved in 0.2M NaOH to a concentration of 10mM. The solution was diluted 1:10 in PBS at a final concentration of 50μM (20μl was added to the cuvette). The presence of NaOH did not affect the final pH of the assay. (-) BOF 4272 was dissolved in distilled water (600ml) at a final concentration of 0.118μM (10μl was added to the cuvette). Amflutizole (3mg) was dissolved in 1ml DMSO and made up to a final volume of 100ml with distilled water at a final concentration of 0.104μM (10μl was added to the cuvette). The percentage of activity which remained after the addition of the inhibitor was calculated.

2.3.4. Determination of NADH oxidase activity on cell surfaces

NADH oxidation was measured spectrophotometrically at 340nm, using an extinction coefficient of $6.22 mM^{-1}cm^{-1}$. Cells were seeded in the four chambered glass slides at $2x10^5$ cells/ml for EA.hy 926 cells and $1.5x10^5$ cells/ml for L929 cells the day before the experiment (Section 2.2). On the day of the experiment, the medium was poured off and the cells were washed twice with prewarmed PBS. Fresh medium (0.9ml) was then added to each chamber. NADH (stock solution 5mM) was added to the chambers to a final concentration of 500μM (100μl). Blanks correspond to medium only. Controls were carried out in bijoux containing cell free medium (0.9ml) and NADH (0.1ml). The chambers and bijoux were incubated at 37°C, humidified with 5% CO_2/95% air (v/v) throughout the experiment. Aliquots (100μl) were taken at different time points and were diluted in the acrylic cuvette to 1ml with prewarmed PBS. The absorbances were read at 340nm. The disappearance of NADH in terms of decrease in absorbance at 340nm was generally followed for 6h, apart for the first experiment in which a 5h assay was used.

2.4. SDS-PAGE and Western blotting

2.4.1. Chemicals and instruments

Acrylamide was obtained from Flowgen Instruments, Staffordshire. BBXO (Biozyme bovine xanthine oxidoreductase) was ordered from Biozyme (Blaenavon, Gwent). Electrode paper and the Multiphor II Electrophoresis System were provided by Pharmacia Biotech (Uppsala, Sweden). Nitrocellulose membranes were obtained from Gelman Sciences. Blocking buffer was ordered as SuperBlock from Pierce & Warriner (Chester). Rabbit anti-human XOR antiserum and affinity purified rabbit polyclonal anti-HXOR antibody were supplied by Dr M. Benboubetra and Richard Bryant (School of Biology and Biochemistry, University of Bath).

2.4.2. SDS-PAGE

SDS-PAGE was based on the method of Laemmli (1970) and vertical slab gels were used. The separating gel consisted of 10%(w/v) acrylamide in 1.5M Tris-HCl, pH 8.8 containing 0.1%(w/v) SDS. The gel was polymerised by the addition of N,N,N',N'-tetramethylethylenediamine (Temed, 0.4µl/ml) and 0.1%(w/v) ammonium persulfate. The stacking gel consisted of 5%(w/v) acrylamide, 1.5M Tris-HCl (pH 6.8), and 0.1%(w/v) SDS, polymerised by the addition of Temed (1µl/ml) and 0.1%(w/v) ammonium persulfate. The cells, prepared as described in Section 2.1.4, were resuspended in phosphate buffer (pH 7.4), to obtain a concentration of $1x10^6$ cells per 50µl buffer. After sonication and ultracentrifugation (Section 2.1.4), sample buffer [62.5mM Tris-HCl (pH 6.8) containing 1%(w/v) SDS, 50%(w/v) glycerol, 10%(v/v) 2-mercaptoethanol, and 0.005%(w/v) bromophenol blue] was added in a ratio of 1:1. These samples (100µl/well), including the molecular markers (2µl/well), BBXOR (Biozyme xanthine oxidoreductase; 10µl/well), and human xanthine oxidoreductase (HXOR, 10µl/well) were boiled for 5min, microcentrifuged and finally loaded onto the gel, and were run at 200V, 80mA using a running buffer [0.192M glycine, 25mM Tris and 0.1%(w/v) SDS; pH 8.3] diluted 1:10 in water. Unless used for electrotransfer, the gels were stained with Coomassie Blue [45%(v/v) methanol, 10%(v/v) acetic acid, and 0.1%(w/v) Coomassie Brilliant Blue], and destained with 5%(v/v) methanol, and 7.5%(v/v) acetic acid for 3h at room temperature.

2.4.3. Western blotting

3MM electrode paper and nitrocellulose, corresponding to the size of the gel, were impregnated with transfer buffer [0.048M Tris, 0.039M glycine, 1.3M SDS, and 5%(v/v) methanol]. The electrode paper, the nitrocellulose and the gel were assembled on the electrophoresis system, taking care to avoid creating any air bubbles. Electrotransfer was carried out at a constant current of 0.8 mA/cm^2 [i.e., current = length (cm) x width (cm) x 0.8], for 1.5h. When the transfer was finished, the gel was stained to check for complete transfer. The nitrocellulose was placed in Ponceau S [0.2%(w/v) in 5%(v/v) trichloroacetic acid] to visualise the bands of proteins. After marking the bands, the membrane was washed with TBS [10mM Tris, 0.9%(w/v) NaCl, pH 7.4] and incubated overnight at 4°C in blocking buffer (SuperBlock) or 2%(w/v) BSA solution in TBS.

For immunoblotting, the membrane was first washed with TBS-Tween [TBS containing 0.05% Tween 20]. The primary antibody was then incubated with the nitrocellulose for 2h in a 1:1000 dilution of rabbit anti-human XOR antiserum or affinity purified rabbit polyclonal anti-HXOR antibody in TBS-Tween (5ml), containing 1%(w/v) BSA. After three washes with TBS-Tween (10ml/wash), the anti-rabbit biotin conjugated antibody [diluted 1:500 in TBS-Tween, containing 1%(w/v) BSA; 5ml] was added and incubated for 1h. The blot was washed three times with TBS-Tween (10ml/wash), and incubated with ExtrAvidin-peroxidase (diluted 1:4000; 5ml) for 1h. The membrane was then washed three times with TBS-Tween (10ml/wash), followed by one wash with TBS. Finally, the antigen was revealed using a peroxide substrate [10ml;16.6%(v/v) 4-chloro-1-naphthol (3mg/ml) solution in methanol, 83.3%(v/v) TBS, and 0.04%(v/v) H_2O_2, 30%(w/w) volume].

2.5. Immunoprecipitation

Protein A-Sepharose CL-4B (50 mg) was first washed with PBS (5ml/wash) before the primary antibody affinity purified rabbit anti-human XOR (100mg) was added and incubated overnight at 4°C. After three washes with fresh PBS (5ml/wash), the cell homogenate (Section 2.1.4; 1.2ml/75 cm^2 flask) was added and the solution was left

rotating overnight at 4°C. Three washes with PBS (5ml/wash) were then carried out and the antigen-protein complex was dissociated by adding a solution (0.5ml) of 6M(w/v) urea, 10% (w/v) SDS and 0.1%(w/v) bromophenol blue in H_2O. The dissociation was carried out for 30min at 4°C, after which insoluble matter was allowed to settle at room temperature for 10min. The supernatant was boiled for 5min and loaded onto a gel for detection of XOR protein (Section 2.4.2). Western blotting was then carried out (Section 2.4.3).

2.6. Sandwich ELISA for determination of XOR protein

2.6.1. Materials and instruments

Eight well racks were obtained from Nunc Inc (Naperville, IL, USA). A Multiskan MCC/340, Labsystem (Finland), was used to read absorbances at 492nm.

2.6.2. Method

ELISA was carried out on cell supernatants using a method developed in this laboratory (Price and Harrison, 1993). Polystyrene wells were coated with affinity purified rabbit polyclonal anti-HXOR antibody (4μg/ml; 100μl/well). After blocking with PBS-Tween [PBS containing 0.05%(w/v) Tween 20] containing 1%(w/v) BSA or casein, for 1.5h at 37°C or overnight at 4°C, the wells were washed three to five times with PBS-Tween (100μl/well; 5min/wash). Purified human milk XOR (100μl/well) was then added to the wells using serial dilutions, ranging from 175ng/ml to 0.62ng/ml, in order to establish a standard curve. Cell supernatant (Section 2.1.4) was added either undiluted or diluted (1:2 and 1:5) at 100μl/well. The second antibody, biotinylated affinity purified rabbit polyclonal anti-HXOR (100μl), was added. After 1h incubation and washing, streptavidin-HRP (100μl) was added for 20min and the wells were washed. The reaction was revealed by using the peroxidase substrate [100μl/well; 1% tetramethylbenzidine in DMSO diluted 1:100 in 0.1M sodium acetate/citric acid buffer, pH 6, and containing H_2O_2 (0.1μl of 30%(w/w) H_2O_2/ml)].

It was then stopped after 10-15 min with 1M H_2S0_4 (50µl/well), and absorbances were read at 492nm in each well.

2.7. Immunohistochemical characterisation and confocal microscopy

2.7.1. Materials and instruments

Affinity purified rabbit anti-human von Willebrand factor (vWF) antibody was provided by Dako (Denmark). Anti-rabbit FITC conjugated antibody came from Jackson Immunoresearch Labs., Inc. (West Grove, PA, USA). Vectshield Mounting medium for fluorescence was obtained from Vector Labs (Peterborough). Affinity purified mouse anti-TNG38 antibody was a gift from Dr George Banting (Department of Biochemistry, University of Bristol) and Dr Barbara Reaves (School of Biology and Biochemistry, University of Bath). Affinity purified rabbit polyclonal anti-bovine milk XOR was obtained from Chemicon (Harrow) and affinity purified rabbit polyclonal anti-HXOR antibodies were prepared by Richard Bryant (School of Biology and Biochemistry, University of Bath). Four chambered polystyrene slides were obtained from Nunc Inc. (Naperville, IL, USA). Images were collected on a confocal laser-scanning microscope: LSM510 with either a x40 1.30 NA or a x63 1.40 NA Apochromatic objective, Carl Zeiss (Welwyn Garden). Where mentioned, some images were also collected either using a laser emission confocal fluorescence cytometer (Bio-rad MRC 500) or a fluorescent microscope (Leica BM IRB). The 488nm and 468nm lines of a laser were used for excitation of FITC and rhodamine respectively.

2.7.2. Characterisation of endothelial cells with von Willebrand factor

Trypsinised cells were added to the chambers at approximately $2x10^5$ cells/ml (1ml/chamber). The cells were left to adhere to the slide for 24h at 37°C, after which time the slide was washed twice with prewarmed PBS (1ml/chamber) and then fixed by treatment for 15min with 5%(v/v) acetic acid / 70 %(v/v) ethanol (1ml/chamber) at -20°C. Another wash with PBS (1ml/well) was carried out before blocking with 3%(v/v)FCS/PBS (1ml/chamber) for 30min and incubating at room temperature.

After blocking, the cells were incubated with affinity purified rabbit anti-human vWF antibody (1ml/chamber; dilution 1:200 in 3%(v/v) FCS/PBS), for 2h at 37°C. The slide was then washed twice in 3%(v/v) FCS/PBS (1ml/chamber), and incubated with anti-rabbit FITC conjugated antibody (1ml/chamber, dilution 1:100 in 3%(v/v) FCS/PBS) for 1h at 37°C. Finally, the cells on the slide were washed three times with PBS (1ml/chamber) and mounted under a coverslip. They were observed using a laser emission confocal fluorescence cytometer (Fig.3.1). Control chambers lacked either primary or conjugated antibodies.

2.7.3. Localisation of XOR protein

Cells were grown to confluence in four chambered polystyrene glass slides at approximately $2x10^5$ cells/ml (1ml/chamber). They were first washed twice with PBS (1ml/chamber) and fixed with 4%(v/v) formaldehyde [PFA in PBS with 0.11% (v/v) 10M NaOH and 0.06% (v/v) concentrated HCl; 1ml/well]. This fixation step was followed by three washes in PBS (1ml/chamber). To permeabilise the cells, a 45min incubation was used with 0.1%(w/v) saponin in PBS (1ml/chamber). Then the first antibody (affinity purified rabbit polyclonal anti-HXOR or mouse monoclonal anti-HXOR antibody) was applied to the cells for 2h [different dilutions were used in PBS containing 0.1%(w/v) saponin, 1%(w/v) BSA and 3%(w/v) NGS; 0.5ml/well]. Before the second incubation, the cells were washed three times in PBS/0.1%(w/v) saponin (1ml/chamber). The second antibody was then applied for 1h: anti-rabbit FITC conjugated or anti-mouse FITC conjugated antibodies [dilution 1/100 in PBS containing 0.1%(w/v) saponin, 3%(w/v) NGS and 1%(w/v) BSA; 0.5ml/well]. Cells were washed three times before being mounted using 10µl of Vectashield Mounting medium over which a coverslip was placed with care to avoid air bubbles. The coverslips were sealed with fingernail vanish and these sealed slides were kept in the dark at 4°C.

For unpermeabilised cells, the same procedure was followed but no saponin was used. In these conditions, cells did not stain when incubated with Golgi marker TGN38 and using anti-mouse rhodamine secondary antibody (Chapter 6). After the fixation step, the first antibody was directly added to the cells.

All the washes were done in PBS only. In all cases, cells were assessed for fluorescence intensity using a laser emission confocal fluorescence cytometer.

2.7.4. Confocal microscopy

The laser was set on 70%. Slides were observed with oil for x400 and x630. The pinhole (small aperture) was adjusted to around one unit. Amplitude gain was adjusted up to three, depending on the sample. Amplitude offset was set at -0.5. Images were first scanned once, then the intensity and the focus were adjusted. Final images were scanned on eight units and saved on the computer linked to the microscope. When necessary the reuse conditions were utilised, so the same conditions were applicable and allowed comparison to controls. When colocalisation studies were done, krypton/argon ion lasers were operated at the same time with excitation of fluorescein at 488nm and of rhodamine at 468nm.

2.8. Heparin-agarose treatment of medium to remove XOR

2.8.1. Materials

Sterile and disposable filters, 0.2μm were obtained from Gelman sciences (Ann Arbor, USA). For medium, see Materials 2.1.1.

2.8.2. Method

A column (3.5cm x 1.5cm) of heparin immobilised on cross-linked 4% beaded agarose (activation: cyanogen bromide) was first washed with 30ml serum free medium (RPMI medium, penicillin/streptavidin). Growth medium (100ml) containing 10%(v/v) FCS was then passed through the column and collected in a sterile container. This medium was then filtered sterile using a 0.2μm filter, and a 10ml syringe. This medium was used for growing cells and was called "XOR free medium". The column was washed with 25mM sodium phosphate buffer, pH 7.4, until the A_{280} reached a baseline level. XOR protein which would have bound to the column was then eluted using 25mM sodium phosphate buffer containing 1 M NaCl.

This protein containing fraction (A_{280}) was assayed for XOR enzymic activity using the fluorimetric method as described in Section 2.3.2.

2.9. Anti-XOR antibodies

2.9.1. Materials

Cyanogen bromide-activated (CNBr) Sepharose B was purchased from Amersham Pharmacia Biotech Ltd. (Herts). Capped tubes were obtained from Bibby Sterilin (Stone, Staffordshire).

2.9.2. Purification of monoclonal anti-XOR antibody

Cyanogen bromide-activated Sepharose B was resuspended in 0.001M HCl (200ml), swirled gently and then left to swell for 15min. The gel was washed on a sintered funnel with 0.001M HCl (600ml), distilled water (600ml), and 0.1M sodium hydrogen carbonate buffer, pH 8.3 (600ml). Protein A (20 mg) was dialysed against several changes of coupling buffer, 0.1M sodium carbonate buffer, pH 8.3, containing 0.5M sodium chloride. The protein A was added to the gel at a ratio of 10mg protein per ml swollen gel (3g Sepharose 4B swelled to approximately 10ml). The ligand and the gel were tumbled end-over-end for 12h at 4°C, in a capped tube. After coupling, the gel was centrifuged at 300 g for 10min; the supernatant containing any uncoupled ligand was retained for a protein estimation. The gel was washed twice with coupling buffer (5ml/wash), by mixing the gel with the buffer and centrifugation. The remaining active groups on the matrix were blocked with 1M ethanolamine, pH 8, for 2h. The gel was washed with three cycles (5ml/cycle) of alternating pH: 0.1 M sodium acetate (containing 0.5M sodium chloride), pH 4, and 0.1M. sodium acetate (containing 0.5M sodium chloride), pH 8. The column (6cm x 1.3cm) was made up using the gel and was washed with PBS containing 0.02%(w/v) sodium azide at 4°C. It was then equilibrated with PBS.

Frozen supernatant from monoclonal clones was thawed and subjected to a protein precipitation with 50%(w/v) ammonium sulphate for 30min. After 15min centrifugation (3000 rpm), the pellet was rinsed twice with PBS (5ml/wash),

resuspended in a few ml of PBS and dialysed against PBS (5l) overnight, at 4°C. The contents of the dialysis tube were centrifuged for 10min at 3000 rpm, before being circulated through the column, overnight. After this binding period, PBS was used to wash off non-specific proteins until the A_{280} was negligible. Bound antibodies were eluted with 4M urea and dialysed overnight against several changes of PBS (2l). The antibody solution (5 to 10ml) was concentrated by dialysis against polyethylene glycol (10g, PEG 20,000) for 1h at 4°C. The specificity of the antibody was checked by using SDS gel electrophoresis (Section 2.4.2) and protein assay (Section 2.2).

2.9.3. Affinity purified rabbit polyclonal anti-XOR antibodies

Unless otherwise mentioned, antibodies used were raised in rabbits and affinity purified on human XOR in our laboratory by Dr Mustapha Benboubetra and Richard Bryant (School of Biology and Biochemistry, University of Bath). The specificity of affinity purified rabbit polyclonal anti-HXOR antibodies was established by Western blotting of human cell extracts (Page *et al*, 1998 and Chapter 5).

2.10. Statistics

For each assay, the mean was calculated as well as the standard error of the mean of at least four determinations. The number of times separate experiment was carried out is expressed by 'n' in the figures.

The Mann Whitney *U* test was used because the sample sizes were too small to support the assumptions of parametric tests such as the Student's *t*-test. Samples were considered significantly different when $P \leq 0.05$ (Siegel, 1956). However, for samples on the borderline, a *t*-test for comparing the mean of two small samples was also carried out. In this case, the difference was considered to be significant when $P \leq 0.01$. Note that the *t*-test assumes that samples are drawn from a normally distributed population (Fowler and Cohen, 1990).

CHAPTER 3
CHARACTERISATION OF EA.hy 926 CELLS AND EXPRESSION OF XANTHINE OXIDOREDUCTASE.

3.1. Introduction

3.1.1. Presence of xanthine oxidoreductase in human cells and tissues

The presence of XOR in endothelial cells was first reported by Jarasch *et al* in 1981. They demonstrated by using immunolocalisation that XOR was present in the capillary endothelial cells of many bovine tissues (liver, heart, intestine, lung). In 1986, Jarasch and coworkers showed the presence of XOR in human endothelial cells. The radioimmunoassay results indicated that levels of the protein were much higher in endothelial cells than in other types of cells. A number of researchers have since sought XOR activity in human cells and the overall results are shown in the following table. It can be seen that the conclusions are highly variable. In some cases, no activity was found in the basal state (Michiels *et al*, 1992; Hassoun *et al*, 1994; Paler-Martinez *et al*, 1994 and Powell, 1995), while in others, activities vary with cell type, and method of assay (Jarasch *et al*, 1981, 1986; Palluy *et al*, 1992; Frederiks *et al*, 1993a, 1993b; Moriwaki *et al*, 1993; Terada *et al*, 1993; Zweier *et al*, 1994, Hellsten *et al*, 1997 and Telfer *et al*, 1997).

Cells/ tissues studied	XOR activity * XOR amount +	Method of detection	References
Bovine EC and tissues.	+ Capillary EC from liver, heart, lung and intestine.	Immunolocalisation.	Jarasch *et al*, 1981
Capillary endothelial cells in bovine and human tissues.	*, + Positive: capillary EC of many tissues.	Histochemistry. Radioimmunoassay.	Jarasch *et al*, 1986.
HUVECs.	* XO activity after hypoxia. Undetectable in basal state.	Fluorimetric assay.	Michiels *et al*, 1992.

HUVECs.	* Hypoxia induces an increase in XO activity and decrease in XDH activity.	Urate assay.	Palluy *et al*, 1992.
Human and rat tissues.	* High activity: EC and Kupffer cells from rat & human liver. Low activity: liver parenchymal cells in rat.	Histochemical assay/ enzymic activity.	Frederiks *et al*, 1993a.
Rat tissues: liver and small intestine.	* Rat liver: very low XO activity, with higher activity in EC. High activity in epithelial layer of small intestine.	Histochemical assay/ enzymic activity.	Frederiks *et al*, 1993b.
Human liver and tissues.	+ Localisation: EC from heart, kidney, brain, aorta, lung, mesentery, hepatic tissue.	Immunohisto--chemical localisation by polyclonal antibodies.	Moriwaki *et al*, 1993.
HUVECs.	* XO + XDH activity detectable. Add IFN-γ, no change seen.	Uric acid formation by HPLC.	Terada *et al*, 1993.
Porcine pulmonary artery EC (PAEC), HUVECs.	* No activity in porcine PAEC and HUVECs even after exposure to hypoxia.	Fluorimetric assay.	Hassoun *et al*, 1994.
Bovine aorta EC, human aorta EC, human coronary artery EC, human microvascular EC, porcine aorta EC, rat aorta EC, rat pulmonary artery EC.	* No activity found except in BAEC and Pulmonary artery EC.	XDH + XO activity: fluorimetric assay.	Paler-Martinez *et al*, 1994.
Human aortic endothelial cells (HAECs).	* XO and XDH activities found.	Spectrophotometric urate assay.	Zweier *et al*, 1994.
HUVECs.	* No activity detectable.	Fluorimetric assay.	Powell 1995.
Human microvascular endothelial cells.	* Increase of XO in human muscle associated with secondary inflammatory processes.	XO activity: chemiluminescence. Distribution: immunohisto--chemistry, monoclonal Ab.	Hellsten *et al*, 1997.
Endothelial cells of blood vessels in placenta.	+ XO was present in cells. No difference in tissues obtained after or before the onset of labour.	Immunostaining with polyclonal antibodies.	Telfer *et al*, 1997

***Table 3.1*: Summary of the studies effected on the expression of XOR in mainly human endothelial cells and tissues.**

3.1.2. The choice of the EA.hy 926 cell line

It was largely the lack of reproducible results (Table 3.1) that led to the work reported in this thesis. The aim was to develop a model human endothelial cell system in which the presence and the role of XOR could be studied quantitatively. In order to investigate the role of XOR in signal transduction, it was convenient to focus on a specific cell type and for these purposes, the immortal human endothelial cell line EA.hy 926 was chosen. This permanent cell line was established in 1983 by Edgell *et al.* It was derived by fusing HUVECs with a human epithelial carcinoma cell line, A549. The resulting hybridoma has been shown to retain a number of properties of the parental endothelial cells including the production of von Willebrand factor, vWF, (Edgell *et al*, 1983). The EA.hy 926 cell line contains measurable XOR, in contrast to HUVECs, in which XOR can only be detected using immunofluorescence and microscopy (Chapter 6). As a cell line, EA.hy 926 cells have the advantages of avoiding the problems posed by primary HUVECs which are difficult to harvest from umbilical cords, prone to infection, have a limited useful passage life and require special growth factors. Finally, there are unacceptable variations in HUVECs from donor to donor, requiring the pooling of several umbilical cords for each population of HUVECs.

3.2. Characterisation of EA.hy 926 cells

The cells were characterised as endothelial cells by using an anti-human vWF antibody. All the cells showed fluorescent staining (Section 2.7.2), as already described by Edgell *et al* in 1983 (Fig.3.1). vWF is an adhesive protein synthesised by endothelial cells. In endothelial cells, vWF undergoes several post-translational modifications. It is secreted constitutively or stored in intracellular secretory Weibel Palade bodies (Vischer *et al*, 1995). vWF is released from endothelial cells as very large multimers and circulates in the plasma (Vlot *et al*, 1998).

This cell line also expresses prostacyclin (Suggs *et al*, 1986) and thrombomodulin (Debault *et al*, 1984), and releases of platelet-activating factor (Bussolino *et al*, 1986). In 1988, Emeis and Edgell reported the fibrinolytic characteristics of EA.hy 926 cells, resembling those of primary cultured human endothelial cells. EA.hy 926 cells are widely accepted as a model for endothelial cells in different areas of research. They have been compared to HUVECs to which they were shown to have similar adhesive responses when stimulated with TNF, but not with IL-4 or IFN (Thornhill *et al*, 1983). When endothelin converting enzyme was purified from EA.hy 926 cells, the enzyme kept the properties of the primary cell line (Ahn *et al*, 1995). The cell line was also used as a model for studying endothelial cell-induced modification of LDL (low density lipoprotein), in which respect there were no significant differences from HUVECs (Pechamselin *et al*, 1996). However, in 1997, Claise and coworkers showed that the antioxidant enzyme activities of superoxide dismutase (SOD), catalase and glutathione peroxidase were significantly lower (respectively 54%, 71% and 8% of levels in HUVECs) in EA.hy 926 cells compared with HUVECs. These lower antioxidant defences need not affect our model in which catalase and SOD are of most interest (Chapter 5).

EA.hy 926 cells show the characteristic appearance of endothelial cells; Fig.3.2 illustrates the cells at day 4. The endothelial cells grow into a single cell confluent monolayer, developing a characteristic "cobblestone" morphology. Their doubling time is 17h ± 1h, as shown in Fig.3.3. The cells were routinely passaged up to a maximum of thirty times, after which fresh cells were used from liquid nitrogen storage.

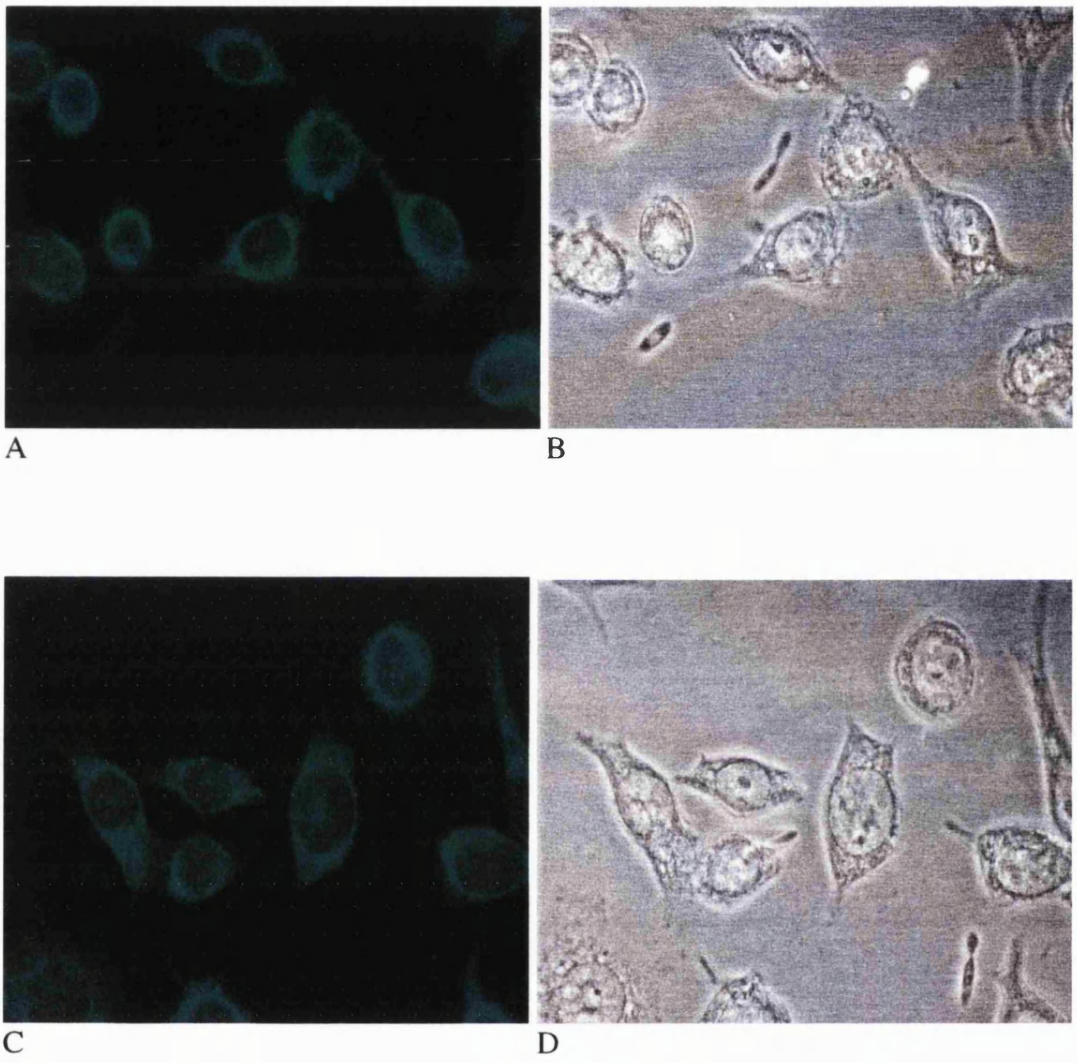


***Fig.3.1**: Characterisation of the EA.hy 926 cells with anti-vWF antibody.*
Cells were permeabilised and fixed by incubation for 15min with 5%(v/v) acetic acid /70%(v/v) ethanol at -20 ℃. Blocking was carried out with 3%(v/v) FCS/PBS. After washing with PBS, anti-vWF antibody was incubated with the cells for 1h (dilution 1:200). Cells were washed with PBS and secondary antibody, FITC conjugated anti-rabbit, was incubated with the cells for 1h (dilution 1:100), (Section 2.7.2). Laser emission confocal cytometer was used, x400. Immunofluorescent images of cells treated as mentioned above (A, C) and differential interference contrast (DIC) images (B, D) are shown.

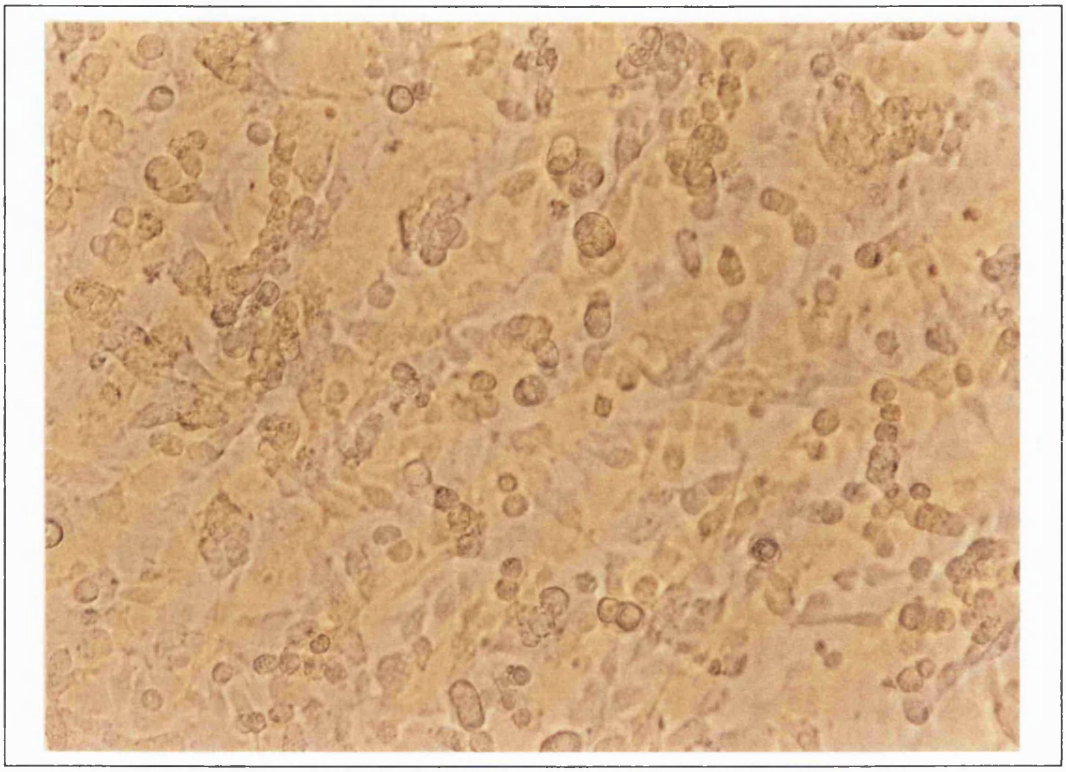


***Fig.3.2*: Morphology of cultured EA.hy 926 cells.**
Cells were set up at $2x10^5$ cells/ml on day 0, and photographed on day 4. (Magnification x 100).

The cell growth pattern (Fig.3.3) shows a lag phase of between zero and two days after which the growth was exponential between 2-5 days. Confluence was achieved on day 8. During stationary phase the number of cells fluctuated until day 14 and even sometimes decreased after day 10. This variation might be due to cell death from overcrowding followed by cell proliferation when space and nutrients are available. The viability of the culture was checked by using trypan blue exclusion (Section 2.1.3). A culture was not used if the viability was below 97%.

3.3. XOR activity in EA.hy 926 cells

Having characterised the cells, the next step was to detect any XOR activity in this cell line. For this purpose, a sensitive fluorimetric assay based on Beckman *et al* (1989) was used. Total XOR activity (oxidase and dehydrogenase forms) was calculated in pmoles/min/mg. As the same time, the number of viable cells was checked by trypan blue exclusion.

3.3.1. General pattern

Fig.3.4 shows the growth profile obtained when the cells were set up at $2x10^5$ cells/ml. The cells grew to form a monolayer reaching confluence after 8 days. XOR activity lagged behind cell growth, first becoming detectable on day 5. After this initial lag phase, the enzyme activity rose steeply and reached a plateau around day 8-9, when the cells are confluent. This plateau is followed by another rise which reached maximal values from day 11. The maximum activity obtained was between 2.5-3 pmoles/min/mg protein. The first experiments were carried out using ultracentrifugation at 500,000g for 10min as in the methodology described by Powell (1995). However, this was considered to be excessive and the speed was reduced to 175,000g for 25min. As shown in Fig.3.4 B, a similar pattern of XOR expression was found. Therefore a centrifugation speed of 175,000g was used for subsequent experiments.

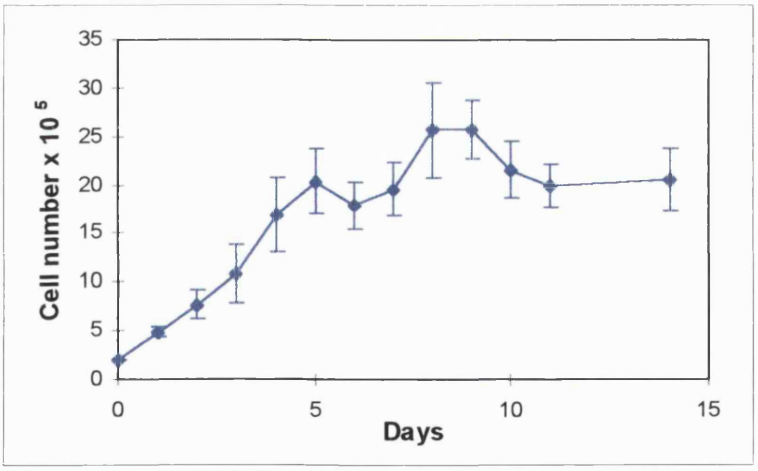
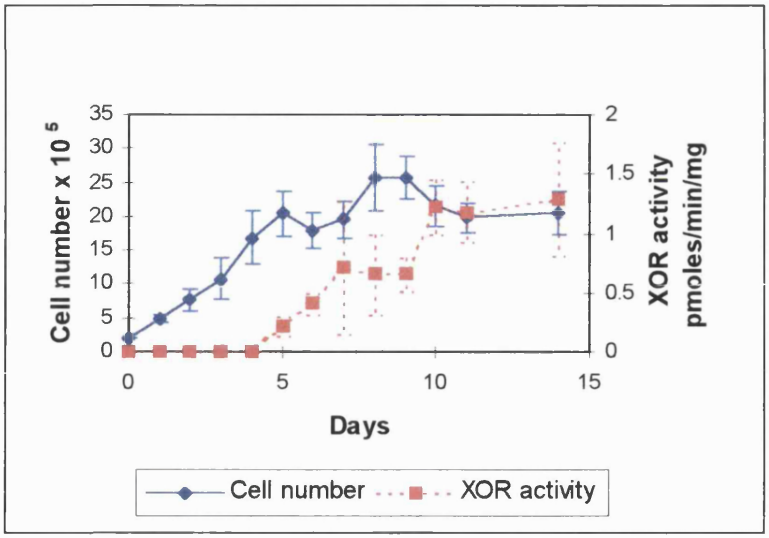


***Fig.3.3**: Growth curve of the EA.hy 926 cell line.*
Every day the cells were counted by trypan blue exclusion. Cell number expressed by n x 10^5 was plotted. Four growth curves, done in duplicate, were plotted together, and error bars were calculated. Values are given as mean ± SEM; n=8.

A)



B)

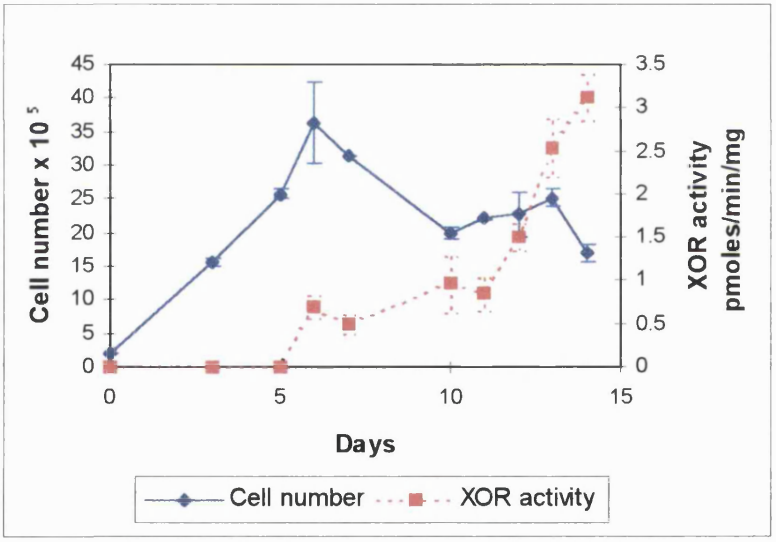


***Fig.3.4**: Expression of XOR in EA.hy 926 cells with time of culture and cell number.*

Every 24h, cells were trypsinised, counted by trypan blue exclusion, then centrifuged (1500 rpm, 5min) and the pellet was resuspended in cell buffer (1.2ml). The cells were sonicated by using a 3mm probe for 20sec and the resulting homogenates were ultracentrifuged for: A) 10min at 100,000 rpm (500,000g), B) 25min at 35,000 rpm (175,000g). Specific XOR activities of cell supernatants were measured on a fluorimeter, in the presence of pterin and methylene blue (Section 2.3.2). Protein assay was also carried out on the cell supernatants (Section 2.2). The specific XOR activity was expressed in pmoles of isoxanthopterin/min/mg protein. Cells were seeded at $2x10^5$ cells/ml on day 0. Every day 2 x 75 cm^2 flasks were assayed for total XOR activity; two samples were analysed per flask. Values are given as mean ± SEM; A n=8; B n=2.

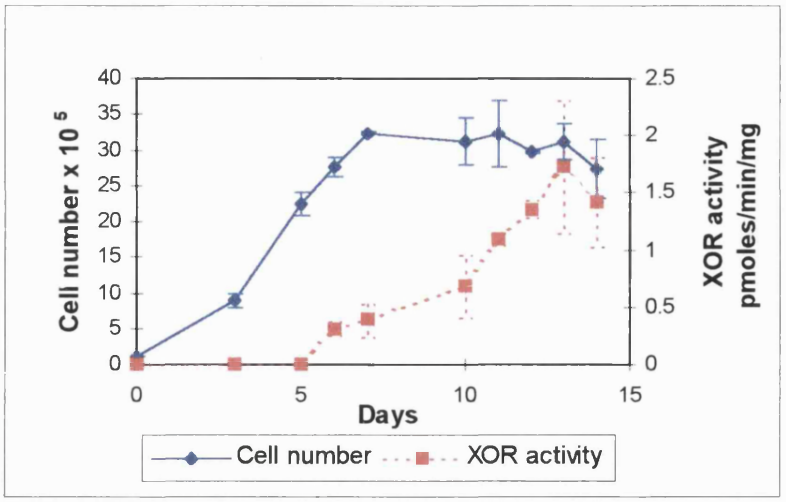
3.3.2. Effects of different cell densities on induction of XOR

In view of the possibility that induction of XOR activity depended on cell density, the expression of XOR activity in EA.hy 926 cells was monitored in cultures with different densities. At the lowest density, $1x10^5$ cells/ml, (Fig.3.5 A) a very similar pattern was found compared to Fig.3.4. The enzyme was absent for the first five days. Its activity then increased to a maximum at a time when the cells are confluent. The plateau is less obvious in this experiment compared to Fig.3.4. A second steep rise then occurs during the time that the cell number is constant and then the activity starts to decrease. It can be noticed that the cell number, for example, rapidly reached the same level as when the cells were set at $2x10^5$ cells/ml, while both the patterns of expression and levels of specific xanthine oxidoreductase activity were comparable. When the cells were set at an higher density, the cell growth pattern appeared to be disturbed. XOR activity was detected earlier (Fig.3.5 B) and on day 3, activity at a relative high level was apparent. The cells did not grow very happily when set at this level, perhaps because of competition for space and nutrients, as they had a tendency to grow on top of each other.

3.3.3. Analysis of XOR activity during the growth cycle

It is possible that XOR activity is present but below the level of detection for this assay. Indeed, this is possibly the case because immunofluorescent techniques (Chapter 6) shows that XOR protein is present on the surface of cells at all stages of the growth cycle looked at. However, though the immunofluorescence shows XOR protein is present, it does not indicate whether or not this protein has enzyme activity. An attempt was made to increase sensitivity of the above XOR assay by increasing the amount of cell protein in the assay. It was found that cells from three $75cm^2$ flasks could be combined, processed and assayed (see above). If more cell protein was used it would have interfered with the measurements. As shown in Fig.3.6, no activity was detectable using the sensitive fluorimetric assay on day 2 with three times the number of cells. On day 3, a very low activity was found in some samples but not in all. Some specific activity was just detectable in every sample on day 4.

A)



B)

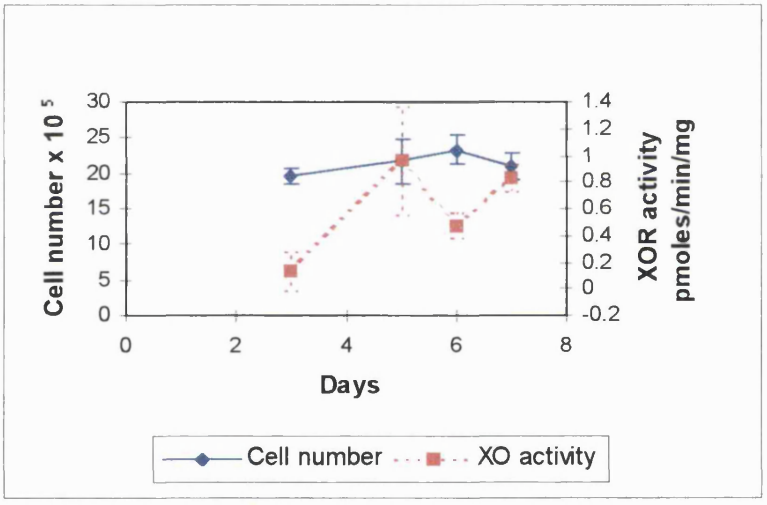


***Fig.3.5**: Expression of XOR at lower and higher density with time of culture and cell number.*

Every 24h, cells were trypsinised, counted by trypan blue exclusion, then centrifuged (1500 rpm, 5min) and the pellet was resuspended in cell buffer (1.2ml). The cells were sonicated by using a 3mm probe for 20sec and the resulting homogenates were ultracentrifuged for 25min at 175,000g. Specific XOR activities of cell supernatants were measured on a fluorimeter, in the presence of pterin and methylene blue (Section 2.3.2). Protein assay was also carried out on the cell supernatants (Section 2.2). The specific XOR activity was expressed in pmoles of isoxanthopterin/min/mg protein. Cells were set up on day 0 at: A) $1x10^5$ cells/ml, B) $3x10^5$ cells/ml.
Every day, 2 x 75 cm^2 flasks were assayed for total XOR activity; two samples were analysed per flask. Values are given as mean ± SEM; A n=2; B n=2.

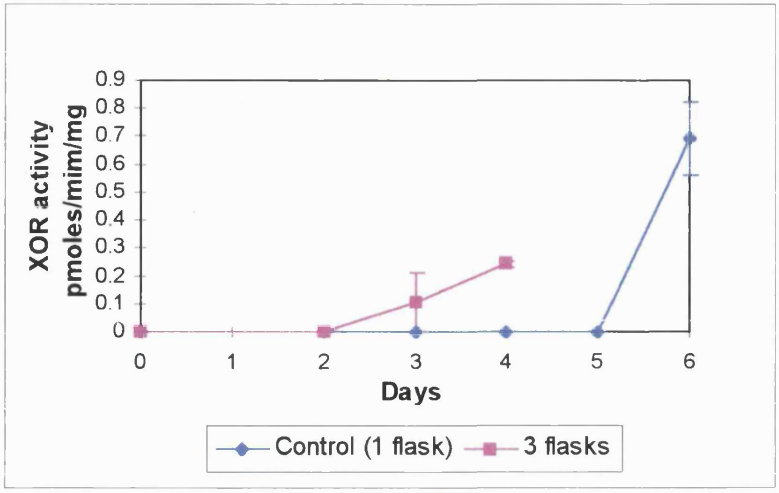


***Fig.3.6**: Expression of XOR activity during the first part of the time course, determined by using higher cell number.*
Every 24h, cells were trypsinised, counted by trypan blue exclusion, then centrifuged (1500 rpm, 5min) and the pellets were put together in the case of the three flasks and were resuspended in cell buffer (1.2ml). The cells were sonicated by using a 3mm probe for 20sec and the resulting homogenates were ultracentrifuged for 25min at 35,000 rpm (175,000g). Specific XOR activities of cell supernatants were measured on a fluorimeter, in the presence of pterin and methylene blue (Section 2.3.2). Protein assay was also carried out on the cell supernatants (Section 2.2). The specific XOR activity was expressed in pmoles of isoxanthopterin/min/mg protein. Cells were seeded at $2x10^5$ cells/ml on day 0, every day 2 x 75 cm^2 flasks were assayed for total XOR activity; two samples were analysed per flask. Values are given as mean ± SEM; n=2.

3.3.4. Effects of trypsinisation of cells on XOR activity

A control was needed to ensure that the XOR expression was not related to the use of trypsin. To examine the possible effect of trypsin on the subsequent expression of XOR in replated cells, the latter were removed by scraping, using a 'rubber policeman'. However the cells grew grossly abnormally each time this approach was tried and eventually this method of cell removal from the tissue culture flask was abandoned. This experiment was unsuccessful in that it was not possible to find an acceptable alternative to trypsinisation for cell removal. EA.hy 926 cells are extremely sensitive to their growth conditions and so they are not amenable to detergent, cold shock or raised ionic strength to remove them from their substratum.

3.4. Conclusions

After investigating different conditions of growth, it was concluded that the best method for measuring specific XOR activity in EA.hy 926 cells was the following, which was used for all measurements of XOR activity in the remaining chapters. Cells were set up at $2x10^5$ on day 0 in a 75 cm^2 flask. On the day required, the cells were trypsinised, counted by trypan blue exclusion, and centrifuged for 5 min at 1500 rpm. The pellet was resuspended in phosphate buffer (1.2ml). The cells were sonicated by using a 3mm probe for 20sec and the resulting homogenates were ultracentrifuged for 25min at 35,000 rpm (175,000g). Specific XOR activity of cell supernatants was measured on a fluorimeter in the presence of pterin and methylene blue. Protein assays were also carried out on the cell supernatants (Section 2.2). The specific XOR activity was expressed in pmoles of isoxanthopterin/min/mg protein.

A characteristic pattern of the expression of XOR in EA.hy 926 cells emerged from the different experiments. XOR activity was not detectable in the initial stages of the growth cycle, up to four days, even when the number of cells assayed was increased three fold. A rise in specific XOR activity was then observed leading to a plateau, when the cells reached confluence. After confluence, a sharp increase in enzyme activity could be observed while the cell number remained stable and eventually decreased. Panus *et al* (1992) reported the expression of XDH and XO activity in cultured BAECs (bovine aorta endothelial cells) and observed a characteristic pattern of total activity under normoxic culture (95% air, 5% CO_2). A single peak of activity was found on day 9 after the cells reached confluence. However, a decrease in specific activity was then observed on postconfluent cells (on days 10, 15, 24 and 30). This result is different to that described above.

The second rise in specific XOR activity, occurring when the cells are confluent, is of particular interest. The relevance and mechanism of this increase was investigated in subsequent experiments (Chapters 4 and 5).

CHAPTER 4
EFFECTS OF CYTOKINES ON XOR ACTIVITY IN EA.hy 926 CELLS.

4.1. Introduction

Cytokines, protein mediators secreted by mononuclear blood cells following stimulation by antigens or other means, can modulate the expression of adhesion molecules on the endothelial cell surface (Pober, 1988). The release of cytokines is preceded by a complex series of events, involving signal transduction, nuclear factor activation and transcription of the various cytokine genes. Interleukins 1 and 6 (Il-1 and Il-6), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) have the most central roles in inflammation (Larrick and Kunkel, 1988). During inflammation processes, endothelial cells are induced to undergo major changes in gene regulation and surface expression of important cell adhesion molecules (CAMs). CAMs mediate the entry of the leucocyte into the inflamed tissue. Neutrophils mediate reactive oxygen metabolites upon activation (Varani and Ward, 1994).

The postulated role of XOR in inflammation suggests the possibility of regulation by cytokines. ROS are produced after human endothelial cells have been subjected to hypoxia/reperfusion and have been implicated in increasing neutrophil adherence (Palluy *et al*, 1992). Cellular levels of XOR activity have recently been shown to be increased by hypoxia in a variety of cells such as bovine pulmonary artery smooth muscle cells (PASMC), bovine pulmonary artery endothelial cells, rat epididymal pad endothelial cells and rat lung (Hassoun *et al*, 1994). Moreover, various cytokines, such as TNF-α; IFN-γ, Il-1 and Il-6 were shown to have a similar effect on bovine renal epithelial cells and on Madin-Darby bovine kidney cells (MDBK) (Pfeffer *et al*, 1994). XOR was also reported to be upregulated by cytokines in endothelial cells: for example, pulmonary endothelial cells from rats (rat lung microvascular cells and pulmonary artery endothelial cells) showed an increase in XOR activity and mRNA in response to IFN-γ (Dupont *et al*, 1992).

It was hoped that EA.hy 926 cells could be used as a model human endothelial cell system in order to investigate the possible roles of XOR in signal transduction leading to cytokine-induced expression of adhesion molecules.

4.2. Methods

Experiments were carried out with TNF-α, IFN-γ and Il-1-β. These were incubated with the cells for 24h at 37°C at final concentrations of 0, 1, 10, 50, 100 and 150 IU/ml. On day 0, cells were seeded at $2x10^5$ cells/ml. The medium was changed every three days. The control cultures contained medium alone which was changed at the same time as that for cells treated with cytokines. Stock solutions of TNF-α, IFN-γ, and Il-1-β were kept at -70°C. The final concentrations were calculated for 30ml of medium from the concentrations of the stock solutions (TNF-α: 40 IU/μl; IFN-γ: 500 IU/μl and Il-1-β: 100 IU/μl). Assays for XOR were done on day 5 and on day 11. The cytokines were added 24h before the assays. These different time points were chosen because day 5 is when XOR activity is first detectable by the fluorimetric assay (see Fig.3.4). The addition of cytokines at that point might induce an early increase in the expression of XOR activity. On the other hand, day 11 corresponds to a situation where the cells are confluent and they show a high level of XOR specific activity. The cells were assayed for XOR activity as described in Sections 2.1.4, 2.3.2 and 2.2. ELISAs were carried out on cells treated with Tumour Necrosis Factor-α (Section 2.6). Mann Whitney *U* tests were used to compare controls and treated cells.

4.3. Results

4.3.1. Effects of Tumour Necrosis Factor-α (TNF-α) on EA.hy 926 cells

This factor is named for its ability to shrink some tumours by reducing their blood supply (Darling and Morgan, 1994). TNF has widespread effects in inflammation, activating macrophages, granulocytes and cytotoxic cells and increasing leucocyte adhesion to endothelial cells (Playfair, 1996).

No significant difference ($P \geq 0.05$) was observed compared to controls when cells treated with TNF-α were assayed for XOR activity either on day 5 (Fig.4.1 A), or on day 11 (Fig.4.1 B). The level of XOR protein was quantified using an ELISA which was carried out on the same samples from day 11. The results did not show any increase in XOR protein (Table 4.1).

4.3.2. Effects of Interferon-γ (IFN-γ) on EA.hy 926 cells

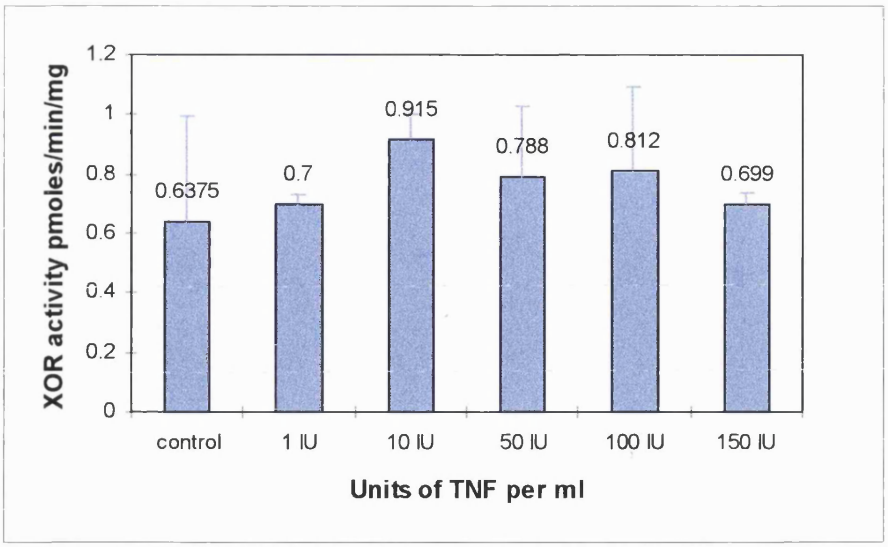
Interferons (IFN) are a group of proteins generated in response to viral infection and also to bacterial lipopolysaccharide. They not only inhibit viral replication but also have a synergistic anti-tumour activity with TNF (Darling and Morgan, 1994), and are more species specific than most other cytokines. IFN-γ is produced by lymphocytes and has a wide range of activities. Its major role is to inhibit viral replication in infected cells and to enhance activity of macrophages. Of interest in the present context is its ability to induce an increase in endothelial cell/lymphocyte adhesion (Roitt *et al*, 1989).

In these experiments the specific XOR activity in cells stimulated by a range of concentrations of IFN-γ was assayed on day 11. No significant increase in activity was seen, as shown in Fig.4.2. When interferon (100IU/ml) was added to the cells and assayed on day 9, there was again no significant difference between the treated and control specific activities, as shown in Table 4.2.

4.3.3. Effects of Interleukin-1-β (IL-1-β) on EA.hy 926 cells

Il-1-β was formerly known as lymphocyte activating factor or endogenous pyrogen (Roitt *et al*, 1989). It has two forms and shares many of its functions with TNF. It is produced by macrophages and induces lymphocyte activation, stimulates macrophages and, of particular relevance to the present study, increases adhesion between leucocytes and endothelial cells. Il-1-β at various concentrations was found to have no significant effect on XOR activity when incubated with EA.hy 926 cells for 24h and assayed on day 11 (Fig.4.3). The specific activities shown in Fig.4.3 are relatively high. It is possible that the XOR activity expressed is influenced by the number of passages the cells have undergone; in this case the cells had a relatively high passage number (approaching 25).

A)



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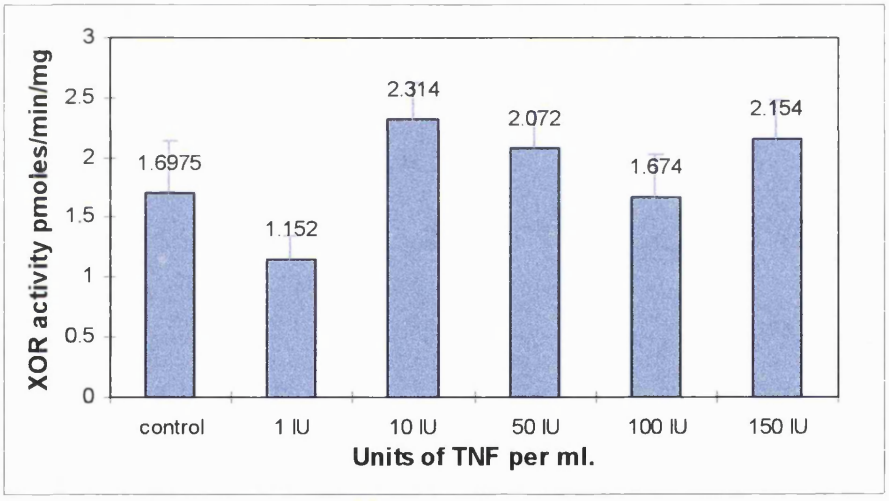


***Fig.4.1**: Effects of TNF-α on XOR activity in EA.hy 926 cells.*
Cells were seeded at $2x10^5$ cells/ml on day 0. TNF-α was added to the cells to final concentrations of 1, 10, 50, 100 and 150 IU/ml. After 24h incubation, the cells were removed from the culture flask by trypsinisation and centrifuged. The cell pellets were resuspended in phosphate buffer, sonicated and ultracentrifuged. The resulting supernatants were assayed fluorimetrically for XOR activity. Protein assays were carried out, and the specific activity was expressed in pmoles/min/mg of protein (Sections 2.1.4, 2.2. and 2.3.2). A) Assays on day 5 B) Assays on day 11.
Values are given as mean ± SEM; n=2.

TNF-α IU/ml.	XOR ng/mg protein.	XOR Activity pmole/min/mg.	Fold increase in XOR activity/control.	Fold increase in XOR protein/control.
control	13	1.69	-	-
1	>12.48	1.152	0.68	0.95
10	9.28	2.31	1.36	0.71
50	11.49	2.072	1.22	0.88
100	>12.51	1.67	0.988	>0.96
150	8.079	2.154	1.27	0.62

***Table 4.1*: Change in XOR activity and protein in TNF-α stimulated EA.hy 926 cells.**
ELISA was carried out on samples as the same time as fluorimetric assays on cell supernatants from day 11 (Section 2.6). n=1.

	Control:	**Interferon-γ:**
Cell number: cells/ml.	$1.86\ 10^6$	$1.68\ 10^6$
XOR activity: pmoles/min/mg.	0.562 +/- 0.127	0.478 +/- 0.156

***Table 4.2*: Effect of IFN-γ (100IU/ml) on day 9.**
IFN-γ was added to the cells at a final concentration of 100 IU/ml. After 24h incubation, the cells were removed from the flask by trypsinisation and centrifuged. The cell pellets were resuspended in phosphate buffer, sonicated and ultracentrifuged. The resulting supernatants were assayed fluorimetrically for XOR activity. Protein assays were carried out, and the specific activity was expressed in pmoles/min/mg of protein (Sections 2.1.4., 2.2.2. and 2.3.2).
Values are given as mean ± SEM; n=2.

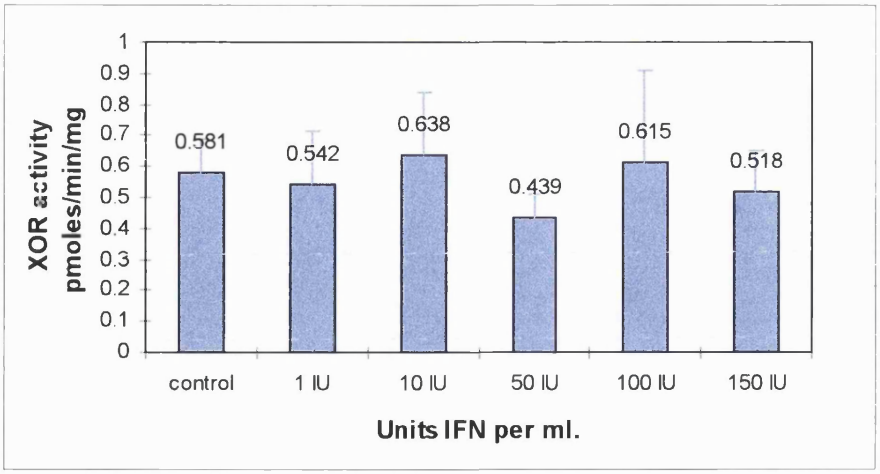


***Fig.4.2**: Effects of IFN-γ on XOR activity in EA.hy 926 cells.*
Cells were seeded at $2x10^5$ cells/ml on day 0. IFN-γ was added to the cells at final concentrations of 1, 10, 50, 100 and 150 IU/ml. On day 11, the cells were removed from the flask by trypsinisation and centrifuged. The cell pellets were resuspended in phosphate buffer, sonicated and ultracentrifuged. The resulting supernatants were assayed fluorimetrically for XOR activity. Protein assays were carried out, and the specific activity was expressed in pmoles/min/mg of protein (Sections 2.1.4, 2.2 and 2.3.2). Values are given as mean ± SEM; n=2.

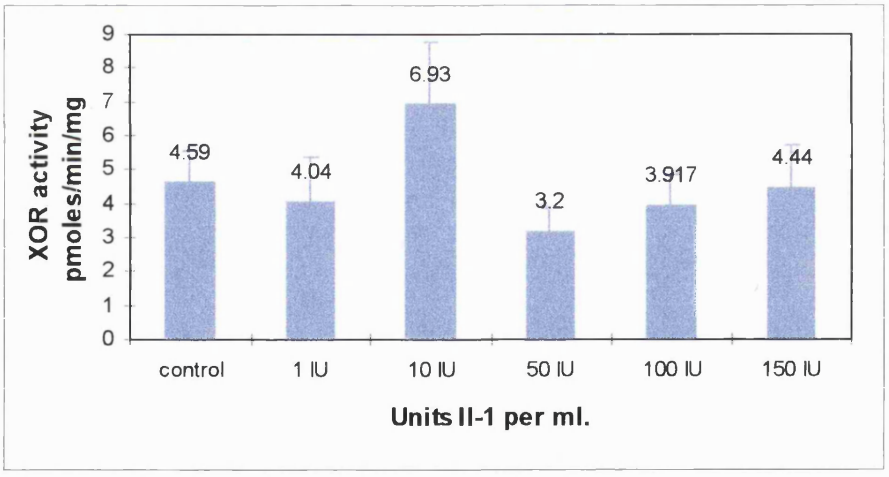


***Fig.4.3**: Effects of Il-1-β on XOR activity in EA.hy 926 cells.*
Cells were seeded at $2x10^5$ cells/ml on day 0. Il-1 was added to the cells at final concentrations of 1, 10, 50, 100 and 150 IU/ml. On day 11, the cells were removed from the flask by trypsinisation and centrifuged. The cell pellets were resuspended in phosphate buffer, sonicated and ultracentrifuged. The resulting supernatants were assayed fluorimetrically for XOR activity. Protein assays were carried out, and the specific activity was expressed in pmoles/min/mg of protein (Sections 2.1.4, 2.2. and 2.3.2). Values are given as mean ± SEM; n=2.

4.4. Conclusions

As mentioned in the introduction to this chapter, XOR activity has been shown to be increased by a range of cytokines in bovine epithelial cells (Pfeffer *et al*, 1994) and by interferon-γ in rat endothelial cells (Dupont *et al*, 1992). Moreover, the results from our own laboratory show cytokine stimulation of XOR in a human mammary epithelial cell line (HB4a) particularly in the case of interferon-γ (Page *et al*, 1998). IL-1β, Il-6 and TNF-α all showed one to two fold enhancement of XOR activity in the HB4a cells, while IFN-γ stimulated by eight to eleven fold.

Similar stimulation might have been expected in EA.hy 926 endothelial cells which would then have served as a model with which to explore links between XOR activity and expression of cell adhesion molecules. The endothelial lining of blood vessels plays the role of "gatekeeper" of the tissues and is a critical focus for the actions of many cytokines. However, neither TNF-α, IFN-γ nor Il-1-β had any significant effect on the expression of XOR activity in EA.hy 926 cells, at any of the cytokine concentrations used in these experiments.

Although the cytokines did not stimulate XOR in these cells, they have been shown to induce CAMs. For example, Mattila *et al* (1992) showed that ICAM-1 (intercellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule), were both upregulated in EA.hy 926 cells stimulated by TNF-α. While these findings were not checked in the present work, the overall implication is that XOR is not involved in cytokine-induced upregulation of CAMs in EA.hy 926 cells.

CHAPTER 5
ROLES OF XOR IN EA.hy 926 CELLS.

5.1. Introduction

The interaction of XOR with an inhibitor is interesting as an aspect of the mechanism of enzyme reaction but also for its medical implications. As XOR is involved in urate production, the inhibition of the enzyme is useful for treatment of hyperuricemia and gout, as well as of post-ischaemic injury. Allopurinol is the best known inhibitor of XOR. It has been used as an effective remedy for hyperuricemia and gout. The inhibition is time dependent and is due to oxypurinol, the oxidation product of allopurinol, which binds stoichiometrically and with high affinity to the reduced molybdenum site (Massey *et al*, 1970). In 1991, a potent new inhibitor was found which inhibits liver xanthine oxidase *in vivo* more effectively than does allopurinol (Sato *et al*, 1991). This inhibitor, BOF-4272, does not show a time dependent inhibition and the (-) isomer binds to the enzyme more tightly than the (+) isomer (Okamoto and Nishino, 1995). Amflutizole is also a potent inhibitor of xanthine oxidase, acting non-competitively (Werns *et al*, 1991). The effects of these inhibitors on the growth patterns of EA.hy 926 cells were investigated in attempts to provide clues concerning the role of the enzyme in a cellular system.

Other approaches were used to study the possible role of XOR in intercellular communication. Attempts were made to perturb the growing cells, or their XOR, or both, using agents which would, for example, remove H_2O_2 or superoxide from the medium. Alternatively, the XOR activating "message" molecule might be a metabolic product of the cell growth, and so conditioned medium was used to see if it would alter the expression of XOR within the growth cycle. Likewise, experiments were designed so that the medium was changed frequently in an attempt to prevent the build-up of signal molecules within the medium and thus to lessen any effect such molecules might have on the cells. These experiments are detailed below.

5.2. Methods

5.2.1. Determination of the kinetics of inhibition of XOR

Inhibition of human XOR enzyme (prepared by Dr Sanders, School of Biology and Biochemistry, University of Bath) was first checked spectrophotometrically at 295nm (Section 2.3.3). The initial rate was recorded in a 1ml cuvette containing 50mM Na^+-phosphate buffer (800µl), 100µM xanthine (100µl), 0.5mM NAD (100µl) and human XOR (10µl, 0.3µg/assay). After 2-3 min of a stable rate, 1mM allopurinol (20µl), 11.8µM BOF (-) 4272 (10µl) or 104µM amflutizole (10µl) was added to the cuvette (optimal conditions from Dr Sanders) and the percentage of inhibition calculated.

5.2.2. Effects of inhibitors on EA.hy 926 cells

On day 0, EA.hy 926 cells were seeded at $2x10^5$ cells/ml. Inhibitors were added directly to the medium at the required concentration from day 0, or later where mentioned. At appropriate times, cells were trypsinised, the cell supernatants were prepared as described in Sections 2.1.3 and 2.1.4, and assays of XOR activity were carried out following the procedure described in Section 2.3. Allopurinol was added to the medium to a final concentration of 50µM, amflutizole to a final concentration of 1µM and BOF (-) 4272 to a final concentration of 100nM.

5.2.3. Assay of cell viability

50µM allopurinol was added to the cells from day 0 to day 8 or 11. The inhibition of XOR in the treated or non-treated cells was checked using fluorimetric assays (Section 2.3). The percentage viability of the non treated and treated cells was calculated using the trypan blue exclusion method (Section 2.1.3).

5.2.4. Studies with frequently changed or with conditioned medium

On day 0, EA.hy 926 cells were seeded at $2x10^5$ cells/ml. On the day of the experiment, cells were trypsinised and the supernatants were prepared for XOR fluorimetric assays as detailed in Sections 2.1.4 and 2.3. Cell numbers were counted by trypan blue extraction. For studies with fresh medium, the medium was changed four times a day. The medium of the controls was changed, as usual, every three days.

For the conditioned medium experiment, used medium was stored at 4°C prior to addition to the cells. Day 'n' medium corresponds to a mixture of 50%(v/v) three day old medium from cells reaching day 'n' and 50%(v/v) fresh medium (Section 2.1.2.1).

5.2.5. Addition of catalase, H_2O_2 or SOD

Cells were incubated with medium containing either catalase from *Aspergillus niger* (100IU/ml), different dilutions of a 8.8M (30%w/w) stock solution of H_2O_2, or SOD (120IU/ml).

5.2.6. Statistics

A Mann Whitney *U* test was carried out to compare controls to different samples of treated cells. The difference was considered to be significant when $P \leq 0.05$. For samples on the borderline, a *t*-test for comparing the mean of two small samples was also carried out. In this case, the difference was considered to be significant when $P \leq 0.01$. Note that the *t*-test assumes that samples are drawn from a normally distributed population (Fowler and Cohen, 1990).

5.3. Results

5.3.1. Determination of the kinetics of inhibition of XOR

The different inhibitors were first tested on pure human XOR before being incubated with EA.hy 926 cells (Results 5.3.2). Spectrophotometric assay at 295nm showed BOF (-) 4272 to be the most effective inhibitor (Table 5.1). Incubation of the inhibitors at 37°C prior to addition did reduce, but only slightly, their inhibitory capacities.

5.3.2. Effects of inhibitors on XOR activity in EA.hy 926 cells

The effects of inhibitors on XOR activity are shown in Fig.5.1. It can be seen that allopurinol and BOF (-) 4272 induced significant reduction in enzymic activity by day 12 ($P \leq 0.05$). Amflutizole did not induce any change in XOR activity after being added

Inhibitors:	Stock solution	Final concentration in 1ml cuvette.	% XOR activity remaining after addition of inhibitor.
Allopurinol, 4°C.	1mM	20μM	13.6%
Allopurinol, 37°C.	1mM	20μM	17.4%
Amflutizole, 4°C.	104μM	0.104μM	6.9%
Amflutizole, 37°C.	104μM	0.104μM	13.6%
BOF (-) 4272, 4°C.	11.8μM	0.118μM	6.4%
BOF (-) 4272, 37°C.	11.8μM	0.118μM	6.25%

***Table 5.1*: Percentage inhibition of XOR in the presence of NAD and xanthine.**
Assays were carried out in air saturated 50mM Na^+-phosphate buffer, pH 7.4. Production of urate was followed spectrophotometrically at 295nm, using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. A linear rate was established for 2-3 min before addition of inhibitor solution, which never diluted the cuvette contents more than 2%. Stock solutions of inhibitors were prepared as described in Section 2.3.3. Inhibitors were preincubated for 24h, either at 4°C or at 37°C. Percentage of inhibition is calculated from rates in the presence and absence of inhibitor; n=1.

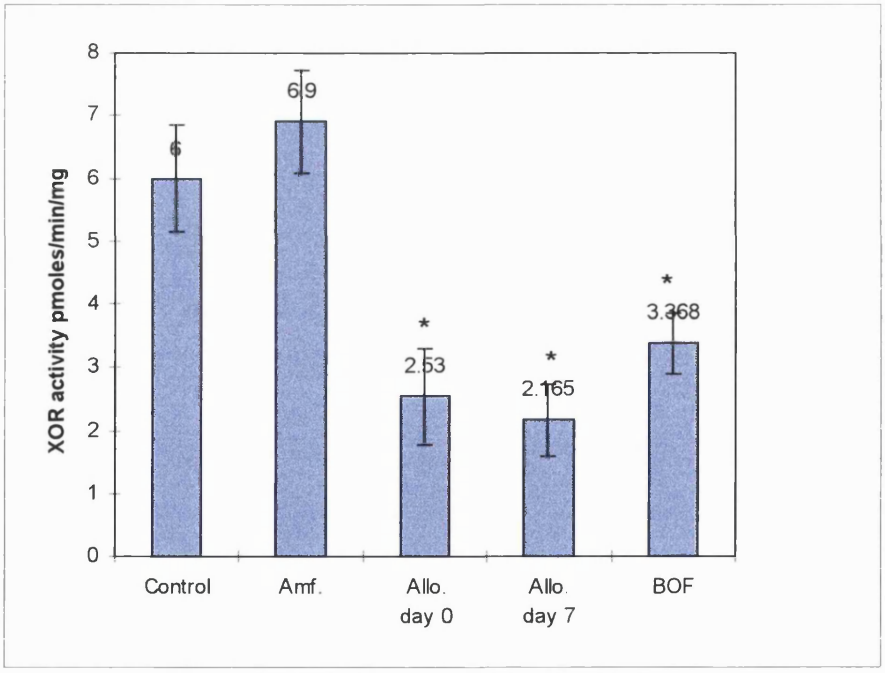


***Fig.5.1*: Effects of inhibitors on XOR activity in EA.hy 926 cells.**
*Inhibitors were added to the medium at the final concentration required on day 0 (and on day 7 for one allopurinol sample): allopurinol 50μM, amflutizole 1μM and BOF (-) 4272 100nM. On day 12, cells were trypsinised, counted, and centrifuged. The cell supernatants were sonicated, ultracentrifuged and the XOR activity was measured (Section 2.3). * represents significant difference ($P \leq 0.05$) using Mann Whitney U test. Values are quoted as mean ± SEM; n=2.*

to the medium for twelve days. This could be due to the inactivation of the inhibitor or to failure of the inhibitor to gain access to the enzyme. Incubation of amflutizole at 37°C for 24h produced a marked decrease in its inhibition of XOR (Table 5.1.); the longer incubation with the cells could have reduced its efficacy still further. As far as access to the enzyme is concerned, subsequent findings (Chapter 6) suggest that at least some of the enzyme would be accessible on the surface of the cell. However, it is not possible to quantify what proportion of the overall activity this represents.

5.3.3. Effects of inhibitors on cell viability

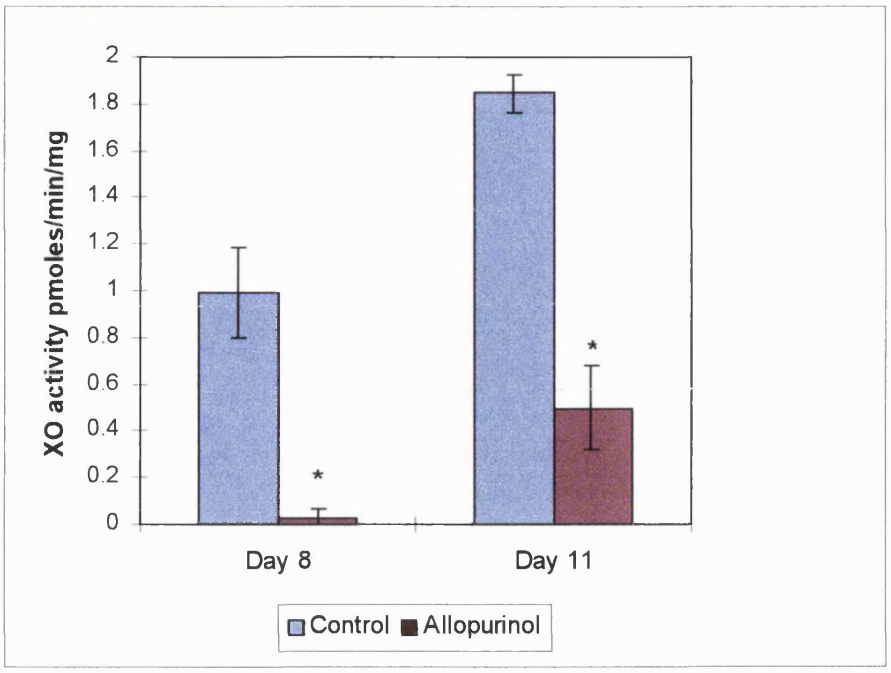
As shown in Fig.5.2 A, allopurinol added from day 0 induced a 90% decrease in specific XOR activity on day 8 and approximately 60% on day 11. These significant results confirm the findings of 5.3.2. However, when cells were counted and the percentages of viability were calculated, no significant difference was found between cells treated with allopurinol and the controls (Fig.5.2 B).

5.3.4. Effects of addition of fresh medium

By changing the medium, the possibility of XOR expression being dependent on signal molecules secreted by the cells was investigated. Fig 5.3 shows that, after 24h, changing the medium four times per day did not significantly affect the expression of XOR in EA.hy 926 cells. However, after 48h of treatment, a decrease in the specific activity of nearly 90% compared to the controls can be seen. These results bordered on significance (P=0.067) with a Man Whitney *U* test, but were significant with a *t*-test ($P \leq 0.01$). It is also interesting to note that the cell numbers were the same in control cultures compared to treated cells (Fig.5.3 A).

Regarding the results described in Fig.5.4 A (after 72h treatment), it should be noted that the cells grew to appreciably greater densities in the situation where the medium was changed four times a day for 72h. At this time, the controls were at a density of $2.1 \times 10^6 \pm 0.22$ cells/ml while the test cells were at a density of $4.14 \times 10^6 \pm 0.76$ cells/ml. This difference is presumably the result of extra nutrients being accessible to the cells in fresh medium. However, the specific XOR activity was found to be 50% lower in these cells compared to controls (Fig.5.4 A) and there was a statistically

A)



B)

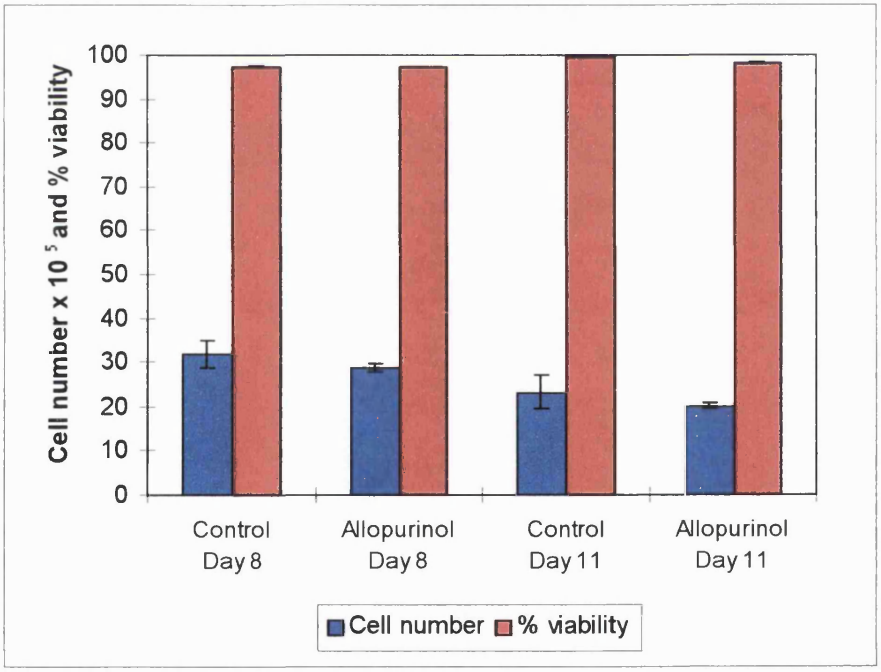
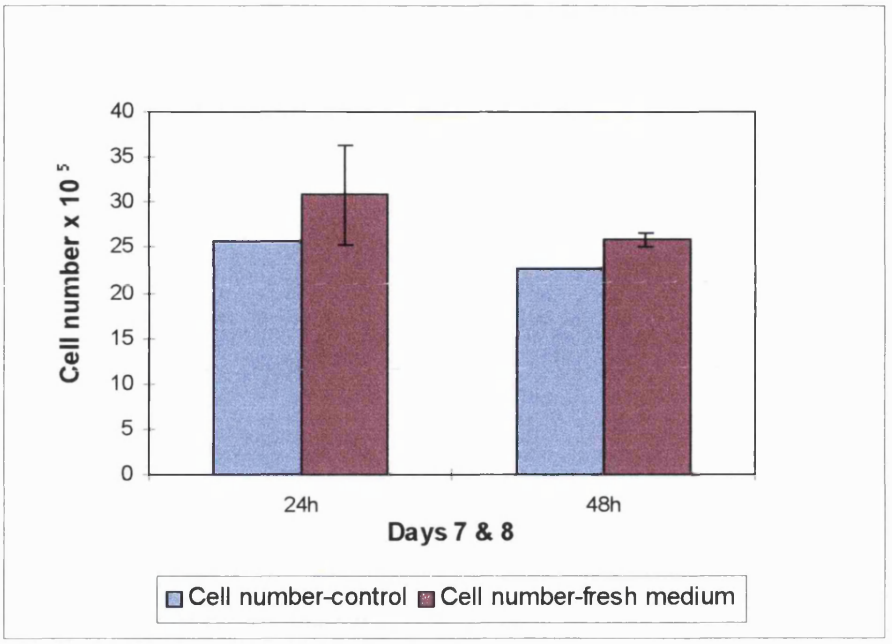


***Fig.5.2**: Effects of allopurinol on XOR activity and percentage of cell viability.* *A final concentration of 50μM of allopurinol was added to the cells from day 0 to the day the cells were harvested (day 8 or day 11). A: XOR activity was determined as described in Section 2.3. B: cell number and % viability by trypan blue exclusion (Section 2.1.3). * represents significant difference ($P \leq 0.05$) using Mann Whitney U test. Values are quoted as mean ± SEM; n=2.*

A)



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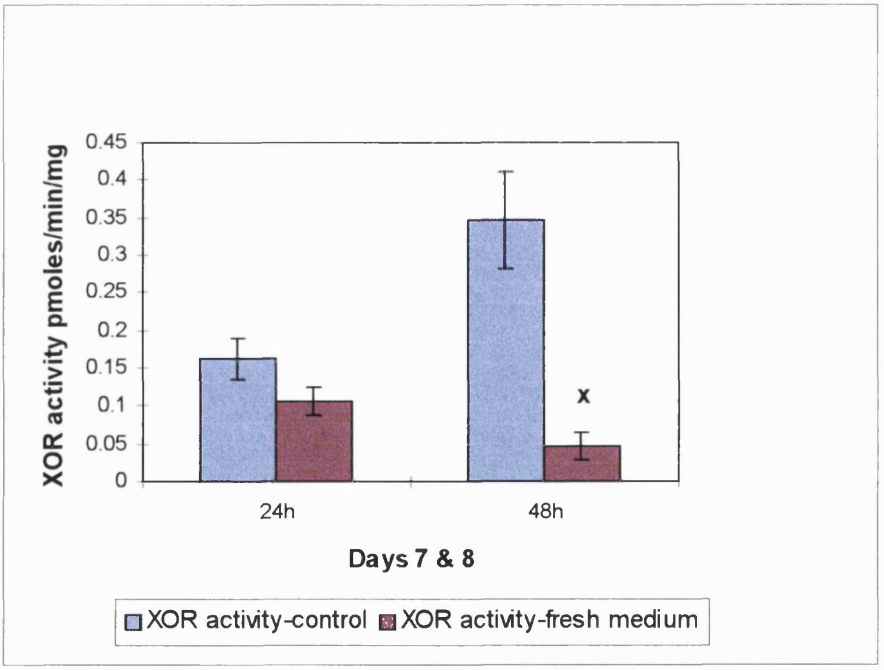
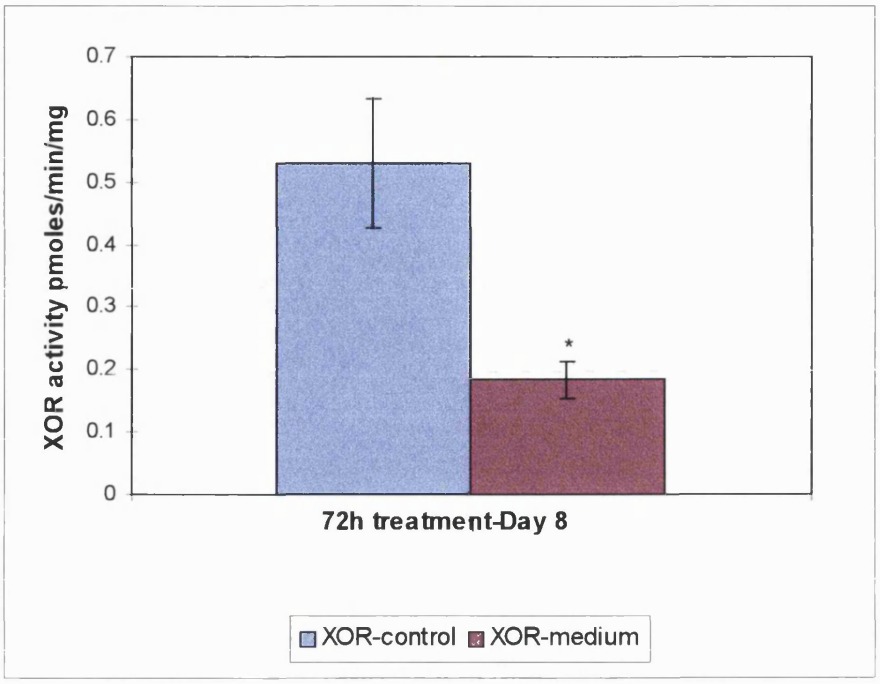


***Fig.5.3*: Effects of changing medium four times per day on cell number and specific XOR activity.**

Cells were set at $2x10^5$ cells/ml on day 0 and medium was changed every three days. From day 6, medium was changed four times a day for 24h or 48h. On the following days (day 7 and 8), the cells were trypsinised and the cell supernatant was prepared for fluorimetric assays (Section 2.1.4 and 2.3). The cell numbers were counted by trypan blue exclusion (Graph A) and the specific XOR activity was calculated (Graph B). ***x*** *represents significant difference ($P \leq 0.01$) using t-test. Values are given as mean ± SEM; n=2.*

A)



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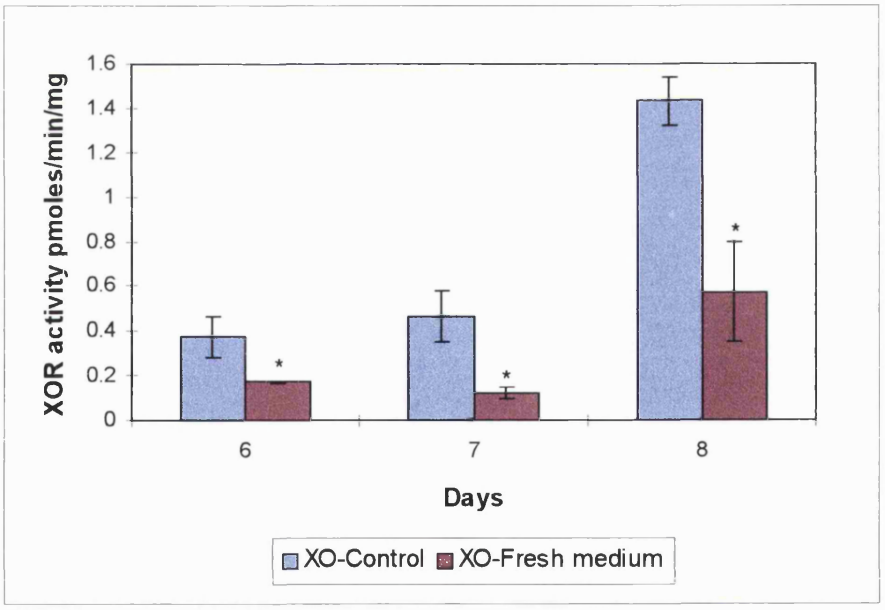


Fig.5.4: ***Time course of XOR expression in cells in which medium has been changed four times a day.***

*Graph A represents cells whose medium has been changed four times a day for 72h, i.e. from day 5 to day 7. Graph B compares the activity in controls to cells whose medium has been changed four times per day on day 4 and 5, before being changed again every three days. Cells were prepared as described in Section 2.1.4 and fluorimetric assays were carried out according to Section 2.3. * represents significant difference ($P \leq 0.05$) using Mann Whitney U test. Values are given as mean ± SEM; n=2.*

significant difference between the two groups. The time course carried out in which medium was changed four times per day for 48h (day 4 and 5) allows us to conclude that an agent implicated in the expression of XOR in EA.hy 926 cells must be present in the medium (Fig.5.4 B). This agent seems to be present at the beginning of the time course. Effectively, when the cells were washed four times a day on days 4 and 5, significant effects on the expression of the specific XOR activity were still visible on day 8, i.e. 72h after the end of the change of medium. The specific XOR activity of the cells treated between day 4 and 5 is approximately half that of the controls (Fig.5.4). The difference between all the controls and the treated cells was significant ($P \leq 0.05$) using Mann Whitney *U* test (day 6, 7 and 8).

5.3.5. The use of conditioned medium to perturb XOR

In Chapter 3, the characteristic growth pattern of EA.hy 926 cells was described. A feature of this pattern is that XOR activity rises to a plateau value, achieved when the cells reach confluence. It then shows a second sharp rise before the cells start to peel off and die (Fig.3.4). Medium from cells producing high levels of XOR at the end of the time course (day 10), was added to cells at the beginning of their time course, at a time where little XOR is normally observed (day 5). Control experiments involved addition of day 5 medium for 24h, 48h or 72h. As shown in Fig.5.5, no differences were observed in cells treated with either day 5 or day 10 medium compared to untreated controls. The cells appeared normal, even after 72h of this treatment. It was found that if conditioned medium was added directly to the cells without dilution by fresh medium then the XOR specific activity was very low and the cells did not look at all happy. Either essential nutrients were missing, or accumulated metabolic products inhibited cell growth. It is for this reason that 50% conditioned medium was always mixed with 50% fresh medium.

5.3.6. Addition of catalase on XOR expression

Catalase was added to the cells to examine the possibility that H_2O_2 could be one agent implicated in the expression of XOR in human endothelial cells. The effects on XOR specific activity in EA.hy 926 cells were sought. As can be seen in Fig.5.6, exogenous catalase did not have an effect on the expression of XOR at the end of the time course after 24h (day 9 or day 11). However, after 48h of catalase treatment, a

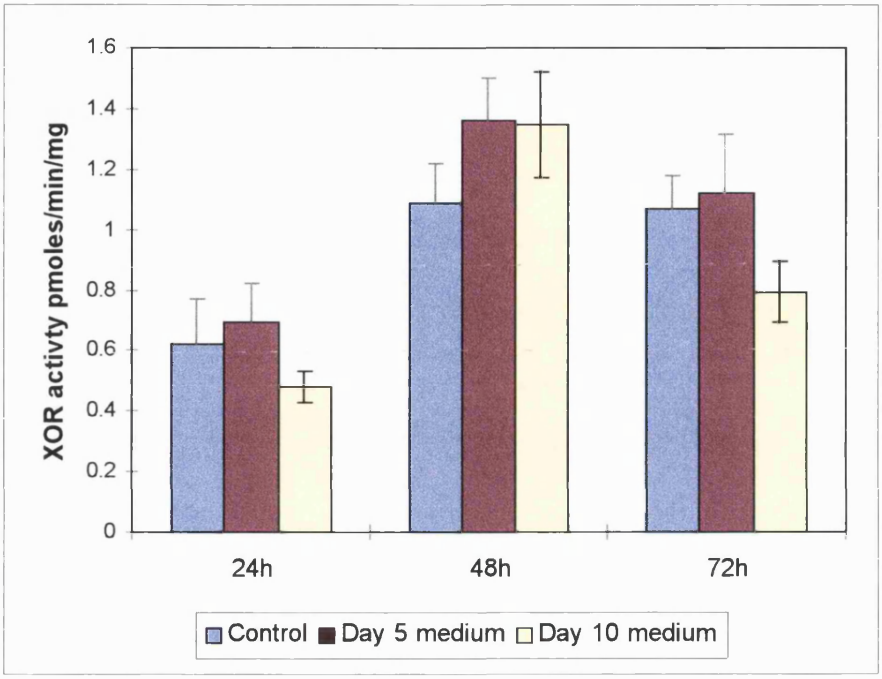
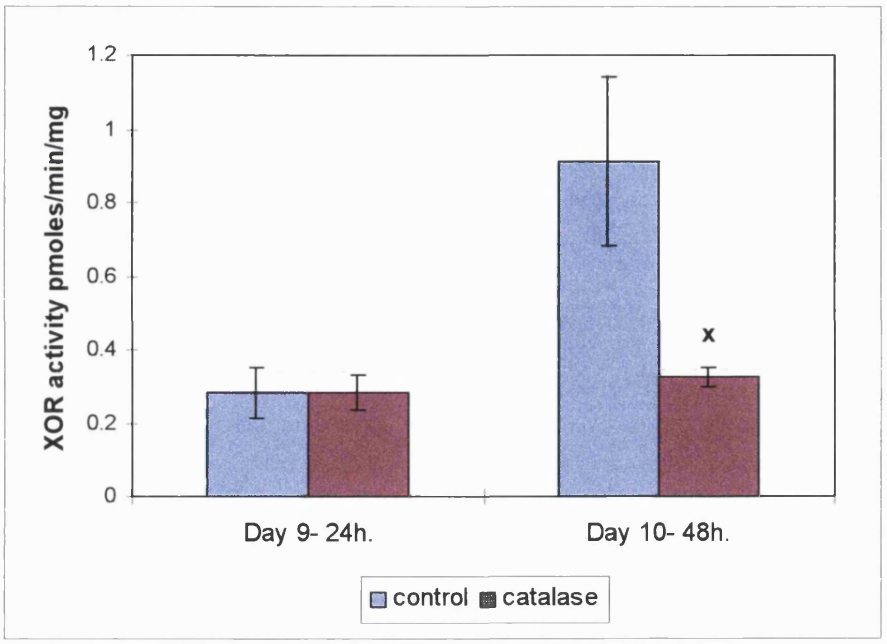


***Fig.5.5*: Conditioned medium experiment: effects of used medium on XOR expression.**
Cells were seeded at $2x10^5$ cells/ml on day 0. The medium of the controls was changed every three days. Day 'n' medium corresponded to cells where medium was replaced on day 5 by a mixture of 50% of fresh medium and 50% of three day old medium from cells reaching day n. The cells were incubated with these different mediums for 24h, 48h, and 72h. Fluorimetric assays were carried out on day 6 (24h), day 7 (48h) and on day 8 (72h) according to Section 2.3. Values are given as mean ± SEM; n=2.

	Controls	Cells treated with catalase
Day 9-24h.	$1.85x10^6 \pm 0.175$	$2.12x10^6 \pm 0.51$
Day 10-48h.	$2.75x10^6 \pm 0.205$	$2.54x10^6 \pm 0.235$
Day 11-24h.	$2.34x10^6 \pm 0.1$	$2.58x10^6 \pm 0.015$
Day 12-48h.	$2.37x10^6 \pm 0.11$	$2.42x10^6 \pm 0.13$

***Table 5.2*: Cell numbers corresponding to the catalase experiments.**
On the day of the assays (see Results, Fig.5.6), cells were counted by trypan blue exclusion (Section 2.1.3). Values are given as mean ± SEM; n=2.

A)



B)

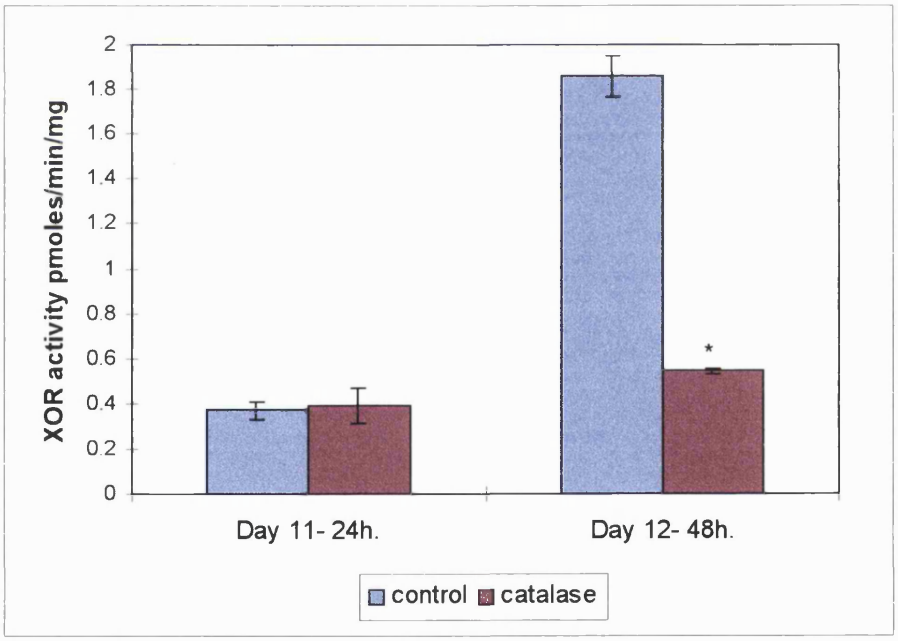


***Fig.5.6**: Effects of the addition of catalase on the expression of XOR in EA.hy 926 cells.*

*Cells were seeded at $2x10^5$ cells/ml on day 0. Catalase was incubated in the medium (100IU/ml) for 24h or 48h. On the day quoted, cells were trypsinised, and prepared for fluorimetric assay (Section 2.3). * ($P \leq 0.05$) and **x** ($P \leq 0.01$) both represent a significant difference using respectively Mann Whitney U test and t-test. Values are given as mean ± SEM; n=2.*

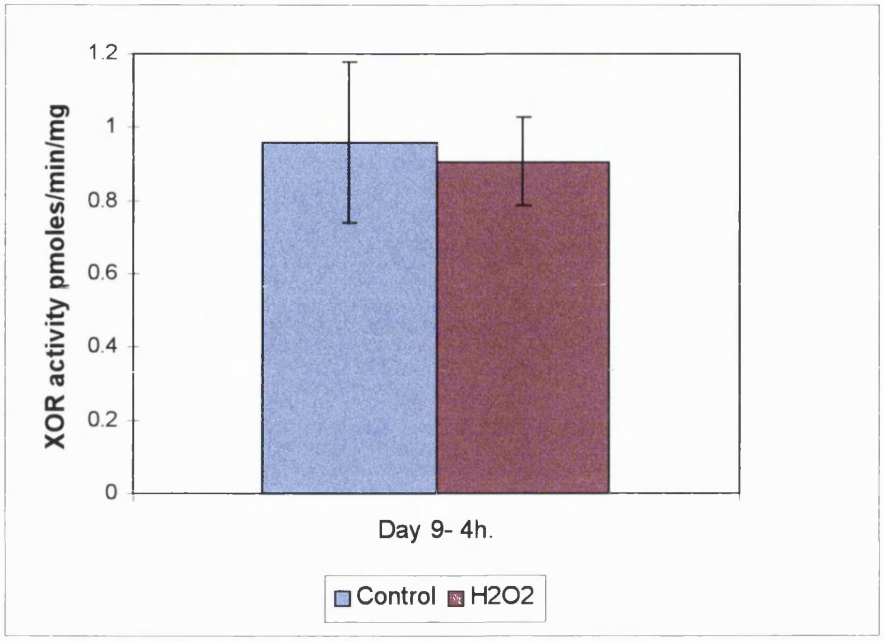
significant decrease of the specific XOR activity in cells treated with catalase was observed. This drop in XOR specific activity on day 12 was significant ($P \leq 0.05$) and on day 10 the significance was borderline ($P=0.067$). However, the latter gave significant result when a *t*-test was used ($P \leq 0.01$). The cell numbers were not significantly different between the controls and the cells treated with catalase and the cells looked totally healthy in the presence of catalase (Table 5.2).

5.3.7. Effects of hydrogen peroxide on XOR expression

Hydrogen peroxide (H_2O_2) is a small, stable molecule that carries no charge and is a potential signalling molecule. H_2O_2 can cross membranes and travel to its target in the cells (Schreck and Bauerle, 1991; Khan and Wilson, 1995). It is produced at sites of inflammation by neutrophils and macrophages and can be detoxified in a single enzymic step, in contrast with superoxide which is predominantly degraded via H_2O_2 (Schmidt *et al*, 1995). Endothelial cells have been shown to synthesise H_2O_2 at an intracellular site accessible to peroxisomal catalase and at a site near the cell surface inaccessible to catalase or gluthione peroxidase (Kinnula, 1992). Catalase was shown to induce a specific decrease of XOR expression in EA.hy 926 cells, and as H_2O_2 is detoxified to H_2O and O_2 by catalase, the effects of adding H_2O_2 directly to the cells were examined. However, a delicate problem was to try to define the concentration of H_2O_2 necessary to induce an effect without damaging or killing the cells.

An 8.8 M (30%, w/w) H_2O_2 stock solution was used. This solution was diluted in PBS to obtain the desired concentration. Trials of different H_2O_2 concentrations were carried out. It was found that in 29mM H_2O_2 the cells were damaged and killed. However, cells grew normally and appeared to be morphologically normal if grown with H_2O_2 at 2.9mM or 5.8mM (maximum values, assuming no loss of H_2O_2). Cells were incubated with 2.9mM or 5.8mM H_2O_2 for 4h, 24h, and 48h (Fig.5.7 and 5.8), but no significant differences were observed in cells treated with H_2O_2 compared to controls. It was thought that the effects of H_2O_2 might have appeared earlier than 24h as H_2O_2 is not a very stable compound in medium because it can be hydrolysed in a variety of ways by the constituents of the medium (Fig. 5.7 A). As shown in Table 5.3, the cell numbers were similar for cells treated with H_2O_2 compared to the controls.

A)



B)

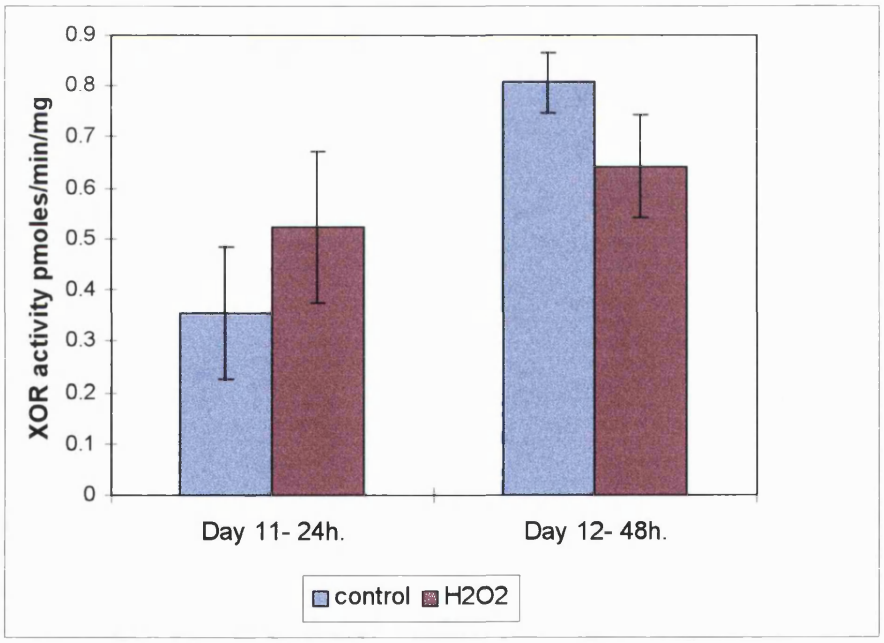


***Fig.5.7*: Effects of the addition of H_2O_2 on the expression of XOR in EA.hy 926 cells.**

Cells were seeded at $2x10^5$ cells/ml on day 0. H_2O_2 from a stock solution of 8.8M was diluted and added to the cells to a final concentration of 2.9mM for 4h, 24h, or 48h on day 9 (Graph A) and on day 10 (Graph B). On the day quoted, cells were trypsinised, and prepared for fluorimetric assay (Section 2.3). Values are given as mean ± SEM; n=2 for A, n=3 for B.

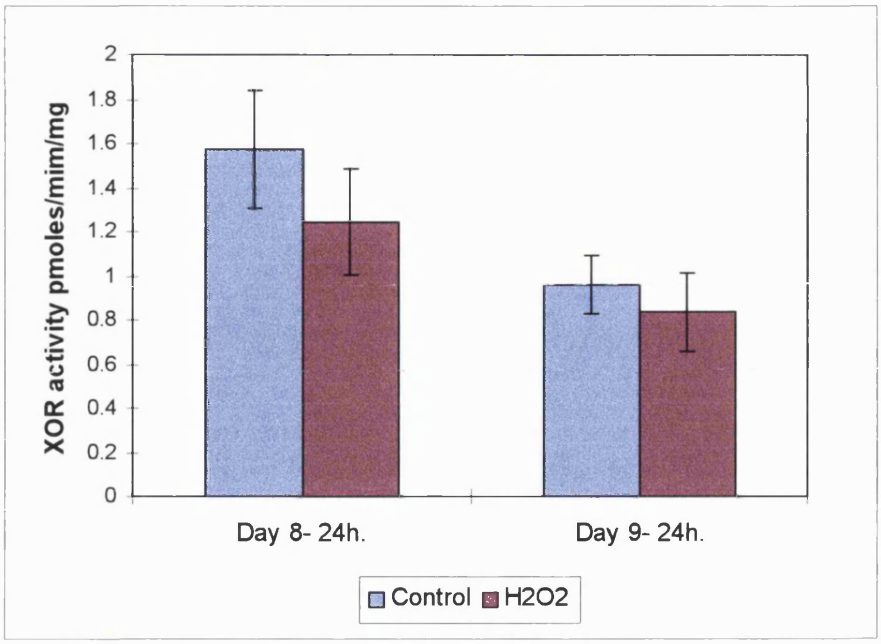


***Fig.5.8*: Effects of the addition of H_2O_2 on the expression of XOR in EA.hy 926 cells.**

Cells were seeded at $2x10^5$ cells/ml on day 0. H_2O_2 from a stock solution of 8.8M was diluted and added to the cells to a final concentration of 2.9mM for day 8 and 5.8mM for day 9. On the day quoted, cells were trypsinised, and prepared for fluorimetric assay (Section 2.3). Values are given as mean ± SEM; n=2.

	Controls.	Cells treated with H_2O_2.
Day 8-24h.	$3.61x10^6 \pm 0.235$	$3.65x10^6 \pm 0.4$
Day 9-4h.	$3.21x10^6 \pm 0.283$	$2.97x10^6 \pm 0.427$
Day 9-24h.	$3.89x10^6 \pm 0.32$	$3.36x10^6 \pm 0.145$
Day 11-24h.	$1.63x10^6 \pm 0.07$	$1.93x10^6 \pm 0.105$
Day 12-48h.	$1.73x10^6 \pm 0.14$	$2.155x10^6 \pm 0.155$

***Table 5.3*: Cell numbers corresponding to the H_2O_2 experiments.**

On the day of the assays (see results, Fig.5.7 and Fig 5.8), cells were counted by trypan blue exclusion (Section 2.1.3). Values are given as mean ± SEM; n=2.

5.3.8. Effects of superoxide dismutase (SOD) on XOR expression

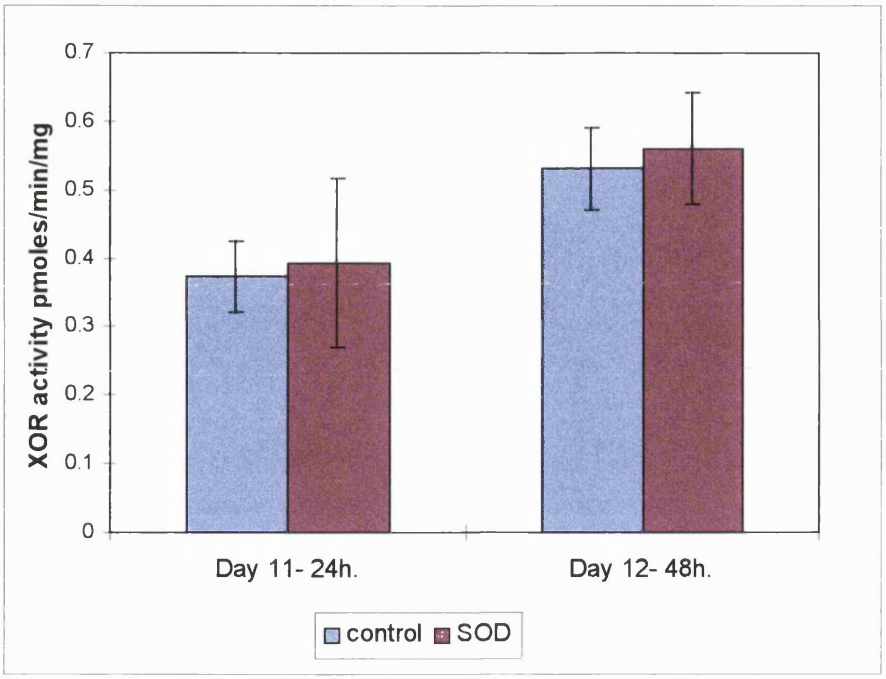
To carry on the investigation after the catalase and hydrogen peroxide results, SOD was added to the cells. An increase in the XOR expression was expected, as SOD produces H_2O_2 while catalase removes it. Superoxide dismutase is a highly specific scavenger of the superoxide radical, preventing reperfusion-injury (Sussman and Bulkley, 1990).

SOD was added at the same point of cell growth as was catalase (Section 5.3.8), i.e. when the cells are showing a second increase in XOR activity having reached confluence. SOD was added directly to the medium for 24h and 48h. No significant differences in results were observed in XOR activity compared to controls (Fig.5.9). As shown in Table 5.4, the cell numbers were similar in controls and cells treated with SOD. No morphological differences were observed between treated cells and controls.

	Controls	Cells treated with SOD
Day 11-24h.	$2.58x10^6 \pm 0.18$	$2.88x10^6 \pm 0.52$
Day 12-48h.	$1.95x10^6 \pm 0.34$	$2.26x10^6 \pm 0.19$
Day 12-24h.	$2.78x10^6 \pm 0.27$	$2.87x10^6 \pm 0.21$
Day 13-48h.	$2.06x10^6 \pm 0.015$	$2.6x10^6 \pm 0.07$

***Table 5.4:* Cell numbers corresponding to the SOD experiments.**
On the day of the assays (see Results, Fig.5.9), cells were counted by trypan blue exclusion (Section 2.1.3). Values are given as mean ± SEM; n=2.

A)



B)

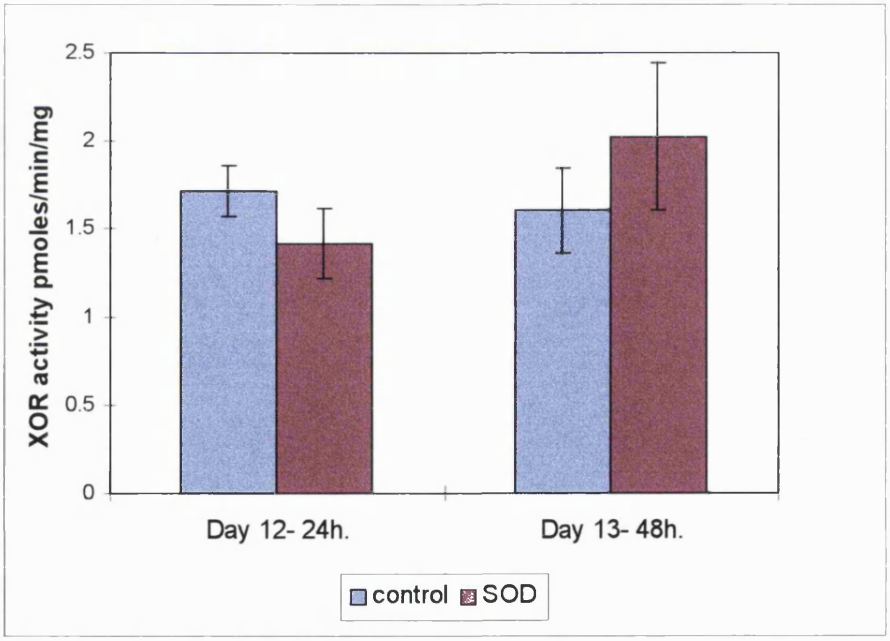


Fig.5.9: ***Effects of the addition of SOD on the expression of XOR in EA.hy 926 cells.***

Cells were seeded at $2x10^5$ cells/ml on day 0. SOD was incubated in the medium (120IU/ml) for 24h or 48h. On the day quoted, cells were trypsinised, and prepared for fluorimetric assay (Section 2.3). Values are given as mean ± SEM; n=2.

5.4. Conclusions

The inhibitors allopurinol, amflutizole and BOF (-) 4272 were shown, as expected, to inhibit purified human XOR. Allopurinol and BOF (-) 4272 also inhibited XOR activity when added to EA.hy 926 cells, confirming the specificity of the pterin assay for this enzymic activity in these cells. On the other hand, amflutizole failed to inhibit XOR activity in EA.hy 926 cells. This could be because of its inability to gain access to the cytosolic enzyme or because of its own inactivation. However, preincubation at 37°C for 24h failed to significantly diminish its inhibitory activity (Table 5.1). Amflutizole has been shown to inhibit cell based XOR activity in other systems. For example, it abolished free radical formation and release in the ischaemic reperfused rat brain. It was previously demonstrated that xanthine oxidase activity did contribute to free radical formation in this model (Phillis *et al*, 1994). Other workers found that amflutizole enhanced ischaemia-induced release of hypoxanthine and suppressed xanthine formation in the rat cerebral-cortex. Cells treated with allopurinol through the time course showed no difference in cell number or viability compared to controls, providing no evidence for an involvement of XOR activity in cell death (O'Regan *et al*, 1994).

The effects of conditioned medium, catalase, hydrogen peroxide and SOD on XOR activity were analysed in detail, in an attempt to determine the role of XOR in signalling. Positive results were found when the medium was changed frequently to prevent build-up of cell secreted products. A similar effect was also found when catalase was present continuously in the medium.

It is interesting to note that, in both cases, it took 48h to show a significant decrease. This might represent the time necessary for the cells to readjust the production of XOR after these different stimuli. On the other hand, the addition of conditioned medium, SOD or H_2O_2 did not induce any observable change in the expression of XOR. Though conditioned medium experiments did not change the expression of XOR, it had to be diluted otherwise essential nutrients would be missing and the cells would die. The same reasoning applied to the concentrations of H_2O_2 used for the peroxide experiments.

All together, these results show that XOR expression can be modified and reduced in EA.hy 926 cells and showed the specificity of the enzymic activity found in this cell line.

CHAPTER 6
DISTRIBUTION OF XOR IN HUMAN ENDOTHELIAL CELLS.

6.1. Introduction

XOR is widely distributed in all human tissue including liver, intestine, heart, brain and in microvascular endothelium (Parks and Granger, 1986; Stevens *et al*, 1991; Xu *et al*, 1994). The localisation of XOR has not been extensively explored because xanthine oxidoreductase activity is extremely low in most of the tissues examined.

The enzyme is generally thought to be present in the cytosol; however there have been only limited publications on the subject of its subcellular localisation. As mentioned in Chapter 3 (Table 1), Jarasch *et al* (1981) described the immunolocalisation of the enzyme in the cytoplasm of bovine capillary endothelial cells. XO was also found to be localised in rat hepatocytes by high resolution immunoelectron microscopy. It was also found that the enzyme was present in the cytosol but not other cell organelles (Ichikawa *et al*, 1992). In 1993, Moriwaki and coworkers purified xanthine oxidase from human liver cytosol. Using this enzyme, a purified antibody was raised which precipitated XO from human liver cytosol. The immunohistochemical localisation of XO in human tissues showed positive results in the cytoplasm of hepatocytes and endothelial lining cells.

In order to determine the detailed subcellular localisation of XOR in cultured endothelial cells, the use of confocal microscopy was combined with classical immunolocalisation (Ojcius *et al*, 1996). In preliminary experiments, specific polyclonal and monoclonal anti-HXOR antibodies were prepared and tested by Western blotting and immunoprecipitation. These antibodies were then used to examine the distribution of the XOR in permeabilised or unpermeabilised EA.hy 926 cells and HUVECs.

6.2. Methods

6.2.1. Validation of specific anti-human XOR antibodies

Cells were seeded at $2x10^5$ cells/ml and were trypsinised between day 10 to day 13. The cell supernatants (referred to as cytoplasmic fractions) were then prepared as in Section 2.1.4 before being subjected to SDS-PAGE. Gels were either stained with Coomassie Blue or transferred to a nitrocellulose membrane for Western blotting, as described in Section 2.4. Supernatants from EA.hy 926 cells were also used for immunoprecipitation. XOR protein was extracted from the supernatants by a protein A-antibody complex (Section 2.5). The binding of the antibody to the protein A was first checked by protein assay (Section 2.2). Removal of XOR protein from the cell supernatant by the protein A-antibody complex was verified by fluorimetric assays (Section 2.3.2) carried out before and after incubation with the complex. Monoclonal antibodies, when used, were purified from hybridoma supernatants as described in 2.9.2. Rabbit polyclonal anti-HXOR was affinity purified as detailed in 2.9.3, using methods detailed in Page *et al* (1998).

6.2.2. Distribution of XOR in human endothelial cells

Permeabilised and unpermeabilised cells were used to localise XOR as described in Section 2.7.3. EA.hy 926 cells were seeded at $2x10^5$ cells/ml, while HUVECs were at $1x10^5$ cells/ml. Initial results (Fig.6.8 and 6.10) were obtained by using a laser emission confocal fluorescence cytometer (Bio-rad MRC 500). Subsequent experiments were carried out with a confocal laser scanning microscope (LSM 510, with either x40 1.30 NA or x63 1.40 NA apochromatic objective: Carl Zeiss). Surface staining was checked on a fluorescent microscope, Leica BM IRB. Affinity purified rabbit polyclonal anti-HXOR and mouse monoclonal anti-HXOR antibodies were used for this study.

6.3. Results

6.3.1. SDS-PAGE and Western blotting

Supernatants from an EA.hy cell homogenate were subjected to SDS-PAGE and Western blotting using rabbit anti-HXOR antiserum or affinity purified rabbit polyclonal anti-HXOR antibodies.

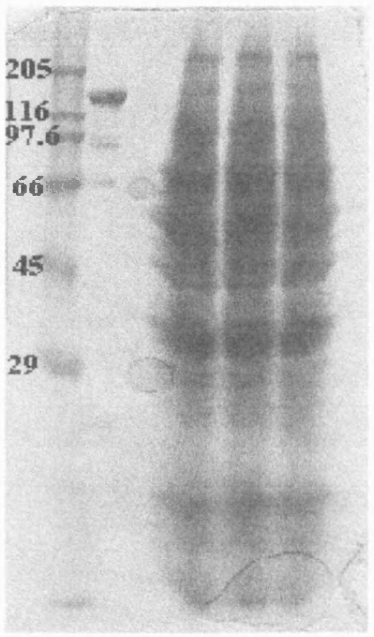


***Fig.6.1*: Gel of EA.hy 926 supernatants.**
Cells were seeded at $2x10^5$ cells/ml on day 0 and were trypsinised on day 13 (Section 2.1.3). The supernatants were prepared for Western blotting as described in Sections 2.1.4 and 2.4, run on a SDS-PAGE gel and stained with Coomassie Blue. Lane 1: molecular weight markers. Lane 2: human XOR prepared from human milk. Lanes 3, 4 and 5: cell supernatant from EA.hy 926 cells.

The characteristic smeared pattern shown in Fig.6.1 was obtained when EA.hy 926 cell supernatants were run on a SDS-PAGE. The XOR activity of the cell supernatant used for this experiment was measured fluorimetrically before the cells were prepared for SDS-PAGE. The specific activity 0.98 ± 0.092 pmoles/min/mg protein is normal for cells at this stage of the time course, as previously shown in Fig.3.4. It is difficult to identify an XOR band in this pool of numerous different proteins. However, a 150 kDa band appears to be present on the gel, at the same level as human XOR band (lane 2). The next step was to optimise the Western blotting method.

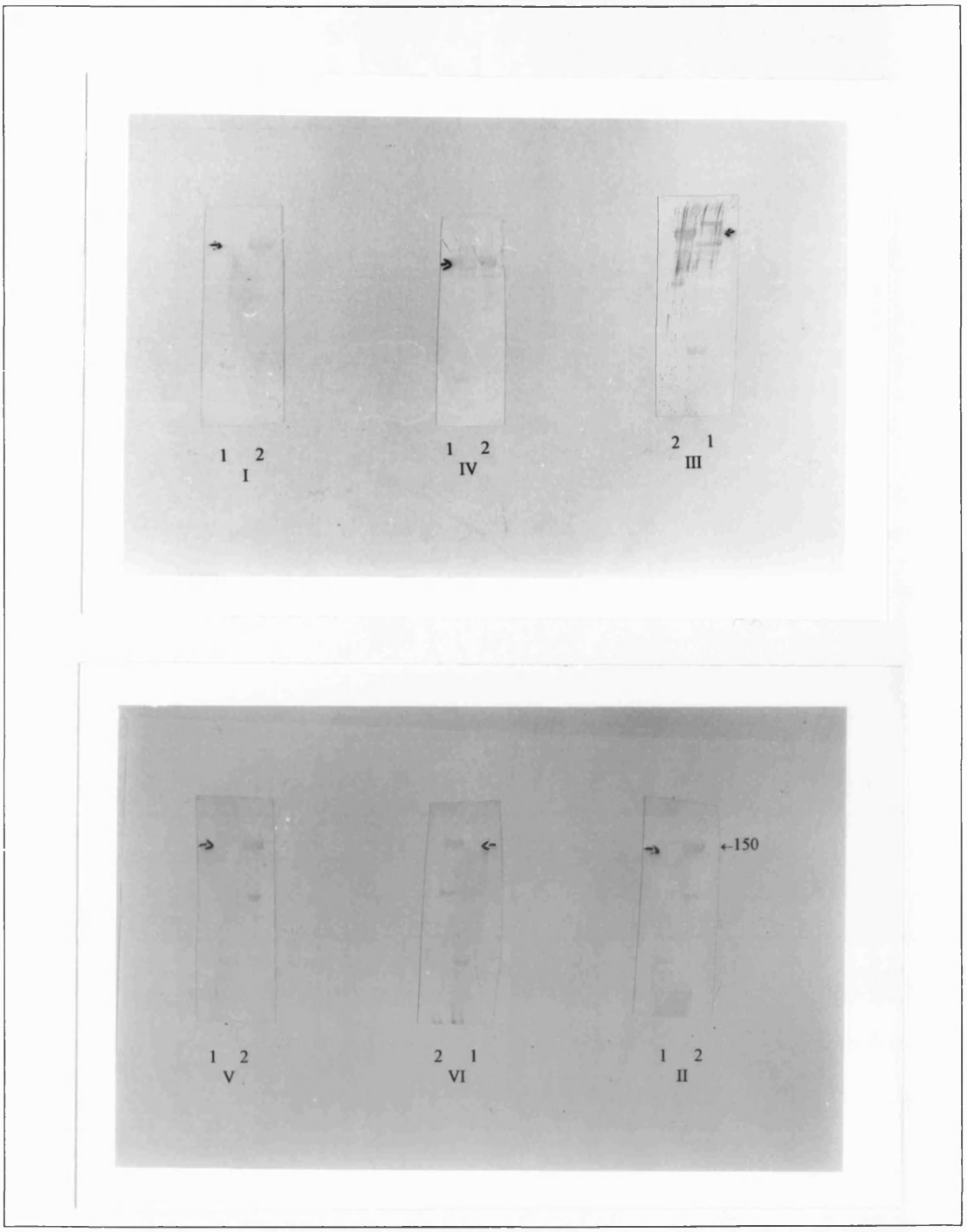


***Fig.6.2*: Different conditions for Western blotting.**
Pure XOR was transferred to nitrocellulose membranes. Lane 1 corresponds to molecular weight markers, lane 2 to human XOR purified from human milk. I) 100μg of biotinylated affinity purified rabbit polyclonal anti-HXOR antibody (1mg/ml), ExtrAvidin peroxidase 1:4000 dilution. II) 100μg of biotinylated affinity purified rabbit polyclonal anti-HXOR antibody (1mg/ml), ExtrAvidin peroxidase 1:2000 dilution. III) Rabbit anti-HXOR antiserum 1:500 dilution, anti-rabbit peroxidase conjugated 1:1000. IV) Rabbit anti-HXOR antiserum 1:500 dilution, anti-rabbit peroxidase conjugated 1:500. V) 50μg of biotinylated affinity purified rabbit polyclonal anti-HXOR antibody (1mg/ml), ExtrAvidin peroxidase 1:4000 dilution. VI) 50μg of biotinylated affinity purified rabbit polyclonal anti-HXOR antibody (1mg/ml), ExtrAvidin peroxidase 1:2000 dilution.

Different conditions for Western blotting were tested with pure human XOR. Fig.6.2 describes the results obtained with purified HXOR using different concentrations of antibodies for Western blotting. The best results (V) were obtained with a biotinylated affinity purified rabbit polyclonal anti-HXOR antibody (50μg) together with ExtrAvidin (1:4000).

The antiserum (III and IV) did not give as clear a 150 kDa band as the biotinylated antibody. Degradation products were picked up in some lanes. This is particularly true for the HXOR lanes of III (antiserum) and of II, V and VI (in which cases the biotinylated antibody was used). Degradation of the protein can only be caused by proteolytic enzymes present in the sample.

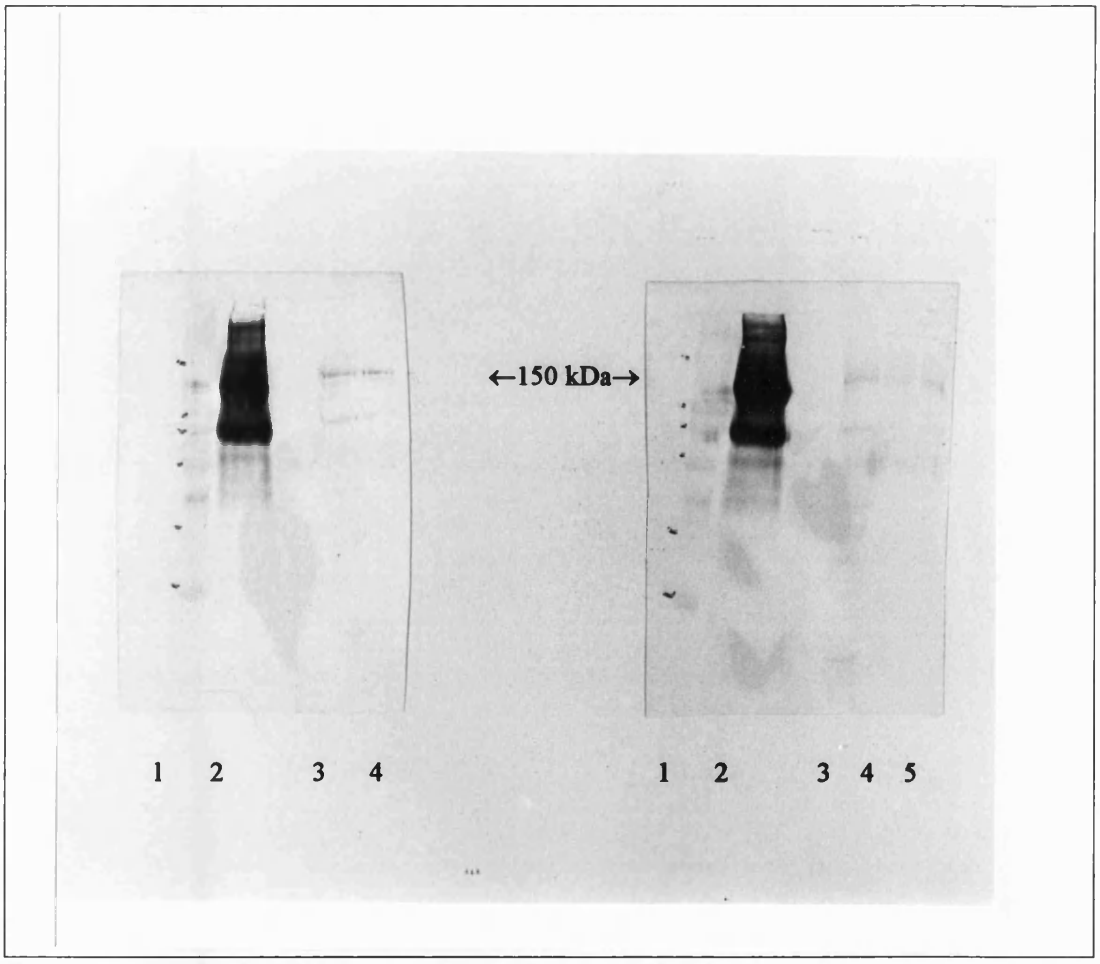


***Fig.6.3*: Western blots.**
For the two blots, rabbit anti-human XOR antiserum (1:1000) and anti-rabbit peroxidase conjugated antibody (1:1000) were used (Section 2.4). Lane 1: molecular weight markers; lane 2: bovine XOR; lane 3: EA.hy 926 cytosolic fraction; lane 4: Buffalo rat liver epithelial (BRLEs) cell supernatant; lane 5: HB4a cell supernatant.

Western blots were then carried out on supernatants from different cell lines. These cell lines are known to express different amounts of XOR activity. BRLEs (rat Buffalo liver epithelial cells) are a clonal cell line described by Coon (1968) which is now thought to be immortalised. These cells show very much higher XOR activity than do EA.hy 926 cells, usually around 77.9 ± 6.60 pmoles/min/mg (Powell, 1995). This is presumably because liver and intestine show relatively high XOR activity (Parks and Granger, 1986; Sarnesto *et al*, 1996). Human mammary epithelial cells (HB4a) show a low XOR specific activity of 0.57 ± 0.09 pmoles/min/mg total protein on day 12 (Page *et al*, 1998). Western blots of BRLEs, HB4a cells and EA.hy 926 cells showed line blots corresponding to a band at 150 kDa, as well as degradation lines. The BRLEs and EA.hy 926 cells gave clearer results than did HB4a cells, probably because of the higher levels of XOR protein in these cell lines compared to HB4a (Fig.6.3).

The quality of the Western blots was generally poor when directly carried out on cell supernatants, even with cell lines expressing higher activity than EA.hy 926 (Fig.6.3, BRLEs).

6.3.2. Immunoprecipitation

To concentrate the XOR protein present in the cytoplasmic fractions of the cells, it was decided to immuno-purify XOR from cells prior to Western blotting. The principle of the method is described in the following figure (Fig.6.4).

The immunoprecipitation technique is based on the affinity of the Fc region of antibodies for protein A, a polypeptide isolated from *Staphylococcus aureus*. The complex formed leaves free the binding domain of the antibody for its antigen, in this case XOR. Therefore, XOR can be concentrated from cell supernatant. The samples obtained were then run on a gel and Western blots were carried out.

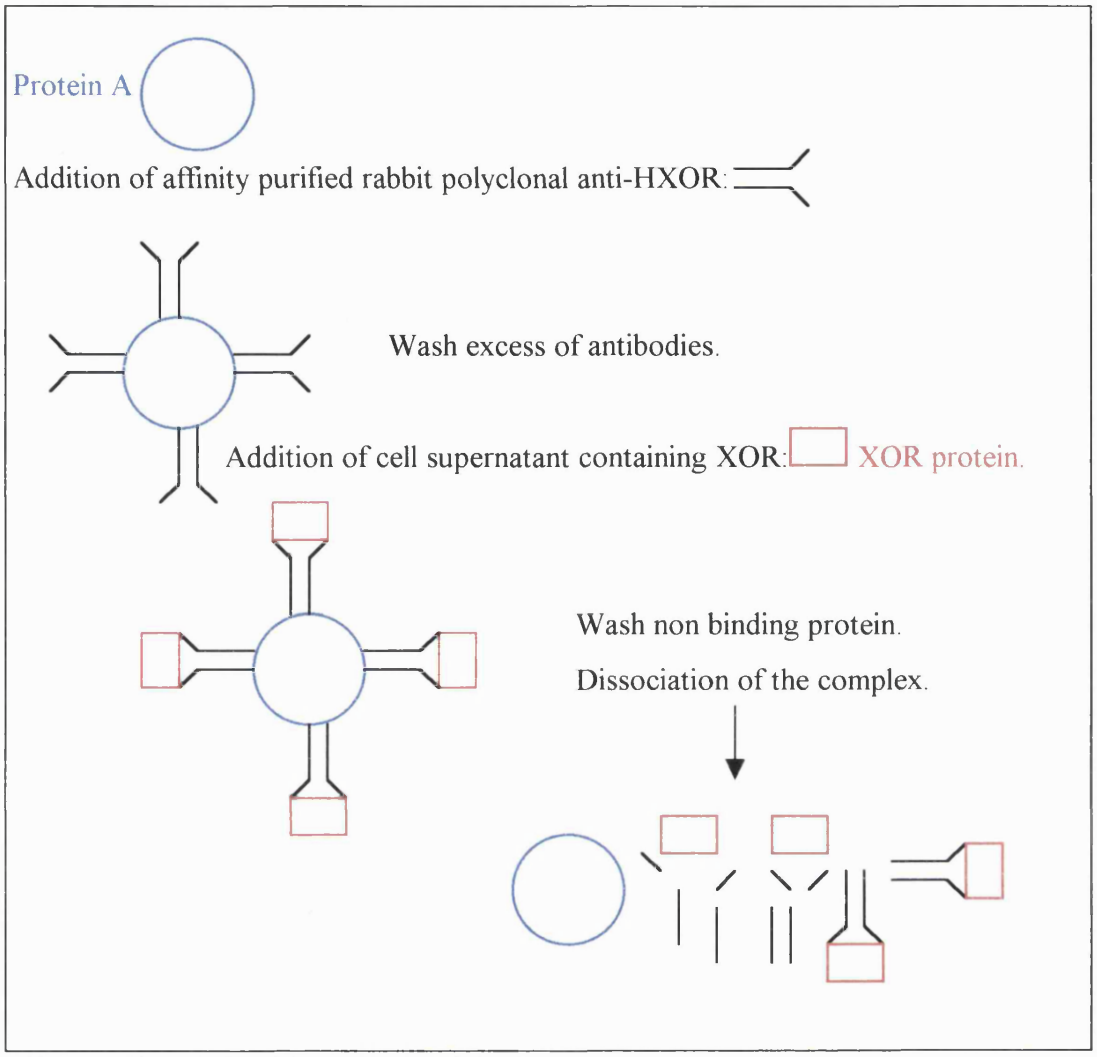


***Fig.6.4*: Principle of immunoprecipitation.**
Affinity purified rabbit polyclonal anti-human XOR antibodies were coupled to Protein A-Sepharose beads by the Fc region. The cells' cytosolic fractions were then added to the Protein A-Antibody complex which removed 100% of the XOR activity in supernatants of EA.hy 926 homogenates. The complex was then dissociated by incubation with SDS and mercaptoethanol, run on a SDS-PAGE gel and Western blotting was carried out.

Two samples of cell supernatants were used to carry out immunoprecipitations (Fig.6.5 and 6.6). The binding of the affinity purified rabbit polyclonal anti-HXOR antibody to protein A was checked after 24h incubation. Protein assays on washes show that only a few proteins were present and that most of the antibodies bound to protein A (Table 6.1).

	1st wash.	2nd wash.	3rd wash.
Samples from Fig.6.5.	0.013mg/ml.	0	0
Samples from Fig.6.6.	0.06mg/ml.	$3.9x10^{-3}$ mg/ml	0

***Table 6.1:** Protein assays on the washes after incubation of the antibodies with protein A.*

The XOR activity of the cell supernatant was checked by fluorimetric assay before addition to the protein A-antibody complex. This activity was compared to the one obtained from the supernatant after 24h incubation. It was found that the affinity purified rabbit polyclonal anti-HXOR antibodies bound to protein A, and removed 100% of the activity present in the cytoplasmic fractions, following immunoprecipitation.

Samples obtained from the immunoprecipitate were run on a gel along with HXOR and molecular weight markers. Some gels were directly stained with Coomassie blue and others were transferred on a nitrocellulose membrane for Western blotting (Section 2.4).

SDS-PAGE of immunoprecipitates (Fig.6.5) shows degradation bands less than 150 kDa in the sample lane (lane 1 and 4), as well as a band corresponding to the heavy chains of rabbit IgG (52 kDa).
Considering the absence of band at 150 KDa, no Western blotting was done on this sample after the immnunoprecipitation. Following the same method, another sample from a cell supernatant was prepared by immunoprecipitation (Fig.6.6).

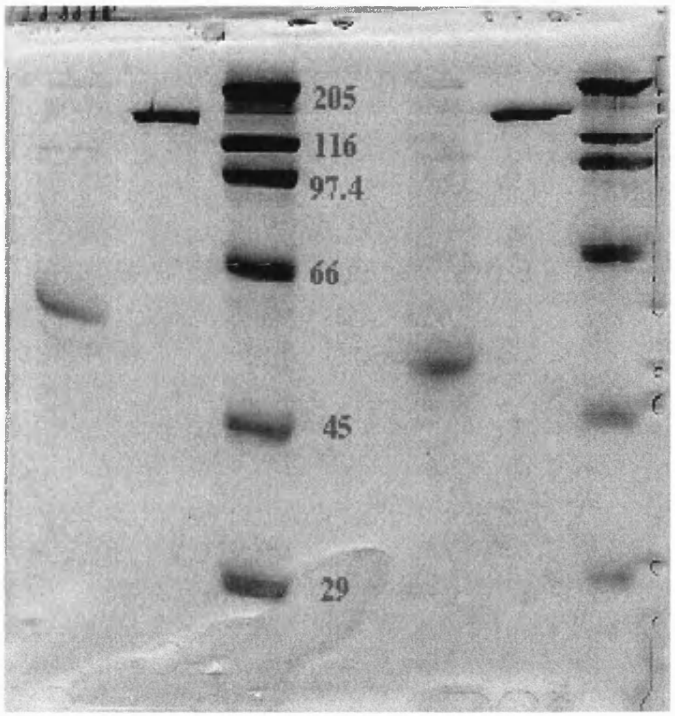


***Fig.6.5*: SDS-PAGE gel after an immunoprecipitation.**
Immunoprecipitation was carried out as described in Section 2.5. Samples obtained (lanes 1 and 4) were run on an SDS-PAGE gel (Section 2.4), which was stained with Coomassie Blue. Lanes 2 and 5 correspond to human XOR (2μg) prepared from human milk. Lanes 3 and 6 are the molecular weight markers.

On the Coomassie gel (Fig.6.6) a band could be observed in the cell supernatant (lane 3) corresponding to 150 kDa. Fig.6.7 illustrates Western blotting on the samples shown in Fig.6.6. Two different Western blots were carried out: one with affinity purified rabbit polyclonal anti-HXOR antibody (Fig.6.7 A) and the other one with mouse monoclonal anti-HXOR antibody (Fig.6.7 B). The mouse antibody was chosen because it should not react with the IgG present on the gel. These IgG's are the affinity purified rabbit polyclonal anti-HXOR antibodies which have been dissociated from protein A (Fig.6.4). This system will give a Western blot, which will not visualise the IgG (heavy and light chains, respectively 52 and 25 kDa).

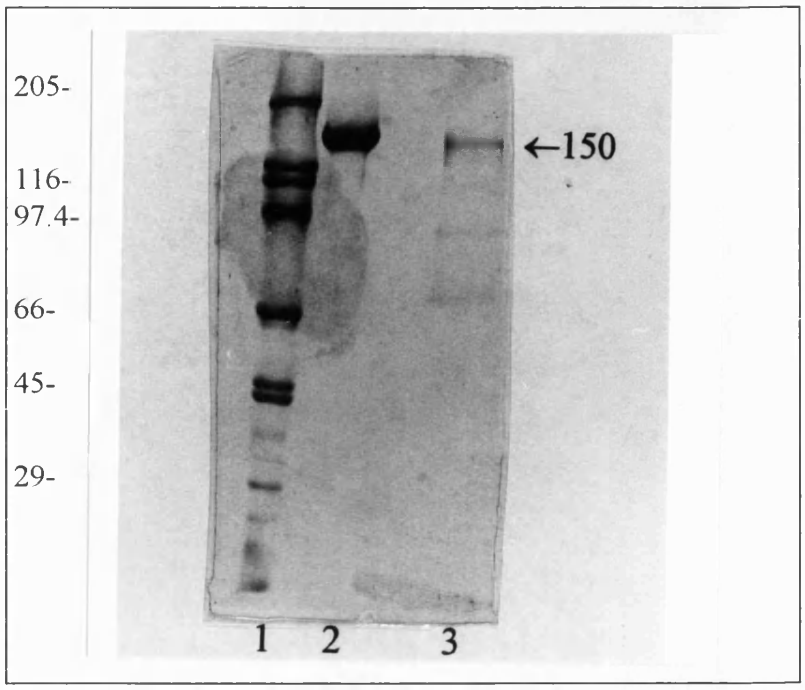


***Fig.6.6*: SDS-PAGE gel after an Immunoprecipitation.**
Immunoprecipitation was carried out as described in Section 2.5 and the samples were run on a SDS-PAGE gel (Section 2.4). Lane 1 corresponds to molecular weight markers. Lane 2 shows human XOR (2μg) prepared from human milk. Lane 3 contains the cytoplasmic samples obtained from EA.hy 926 cells.

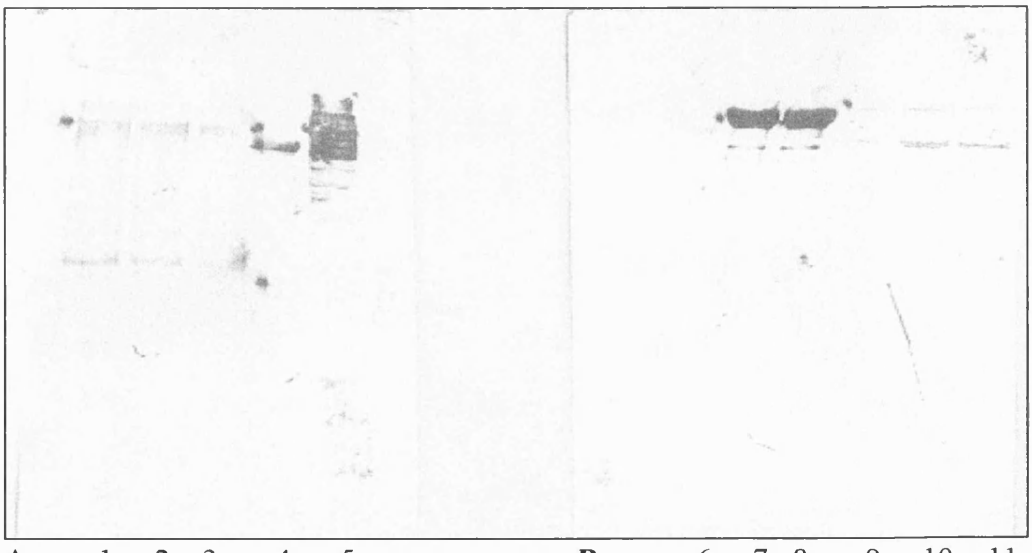


***Fig.6.7*: Western blots after Immunoprecipitation.**
The gel from the samples shown in Fig.6.6 was transferred to nitrocellulose membranes and visualised using: A) biotinylated affinity purified rabbit anti-HXOR antibody (100μg) for 2h, then ExtrAvidin (1/4000) for 1h. B) mouse monoclonal anti-HXOR (200μl) for 2h, then an anti-mouse peroxidase conjugated antibody (1/1000) for 1h. Western blots were revealed by a solution of 4 chloro-1-naphthol containing H_2O_2 (Section 2.4.3). Lanes 1, 2, 3, 9, 10 and 11: cytoplasmic samples from EA.hy cells; lanes 4, 7 and 8: human XOR (2μg) prepared from human milk and lanes 5 and 6: molecular weight markers.

In Fig.6.7 A, a clear band at 150 kDa as well as the IgG band are visible on the nitrocellulose (lanes 1, 2, 3). However, some of the markers also reacted with the biotinylated antibody (lane 5).
The mouse monoclonal antibody reacted only with HXOR. Some degradation products of XOR are visible at the same time as the 150 kDa band (lanes 9, 10 and 11).

6.3.3. Localisation of XOR in human endothelial cells

Two human endothelial cell types, EA.hy 926 cells and primary endothelial cells (HUVECs) were studied to determine the subcellular localisation of XOR. Affinity purified rabbit polyclonal anti-HXOR antibodies were mainly used, along with mouse monoclonal anti-HXOR, as the detection system. With a confocal microscope, the sample analysed is scanned horizontally point after point, line after line, thus defining a field. Each field is analysed by displacing the sample vertically with respect to the objective. The fluorescence emission is detected by a photomultiplier tube, and once converted into an electric current, the signal produces an image on the screen.

6.3.3.1. Localisation of XOR inside human endothelial cells

Cells permeabilised by a gentle detergent, saponin, were incubated with antibodies for the subcellular localisation of XOR.

Initial images clearly show the presence of the XOR enzyme in the cytoplasm of EA.hy 926 cells with a concentration around the perinuclear region (Fig. 6.8). Better quality images [fluorescent images and differential interference contrast (DIC)] were obtained with a confocal laser-scanning microscope (Section 2.7.1 and 2.7.4). The structure of the cells appears more clearly allowing a precise characterisation of the distribution of XOR protein. Fig.6.9 shows the immunolocalisation of XOR in permeabilised EA.hy 926 cells, confirming that the protein is diffusely distributed throughout the cytoplasm, although fluorescence was more intense in the perinuclear region. The outer membrane of nuclear envelope is known to be continuous with the membrane of the endoplasmic reticulum (Loewy *et al*, 1991).

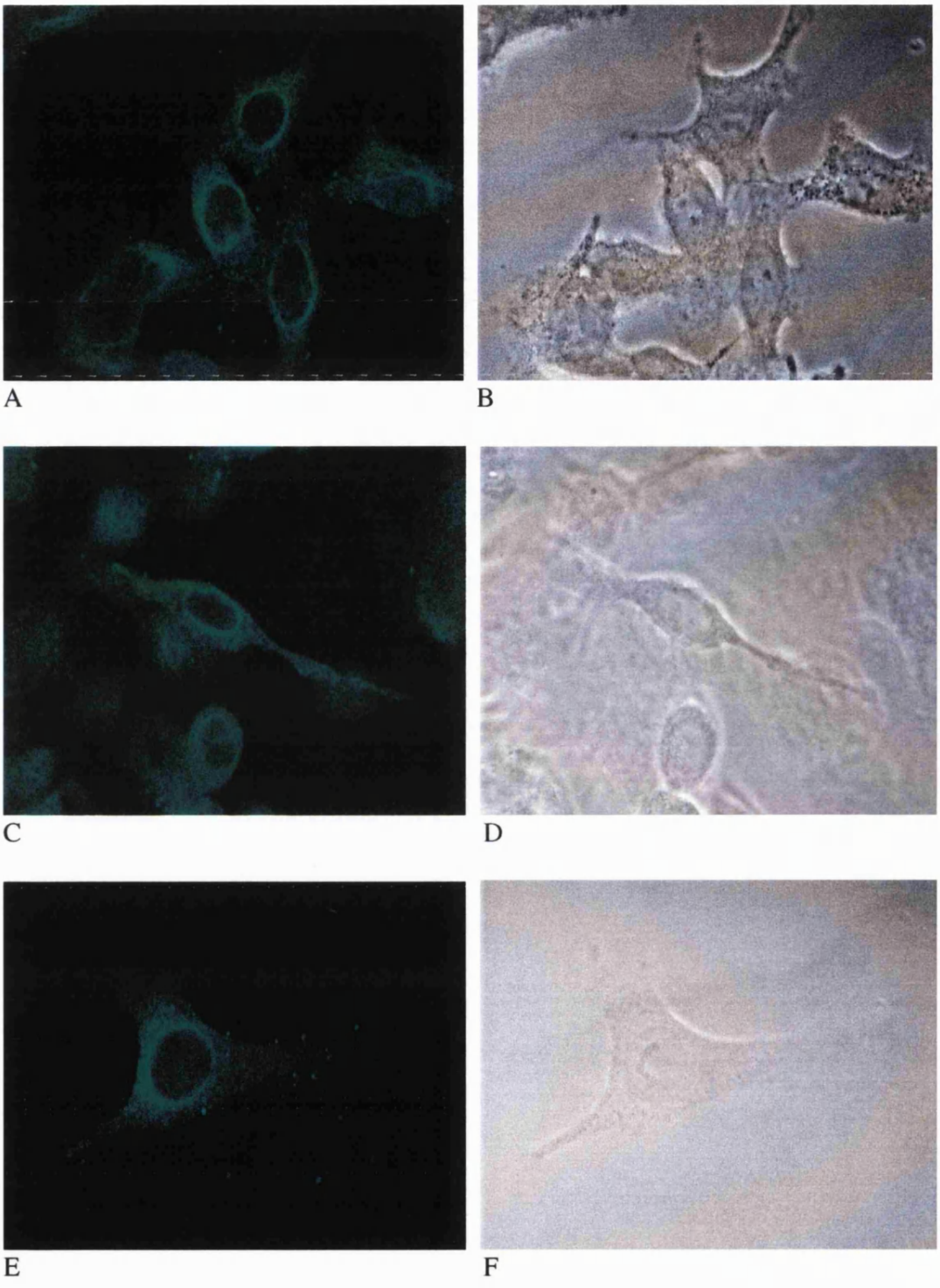
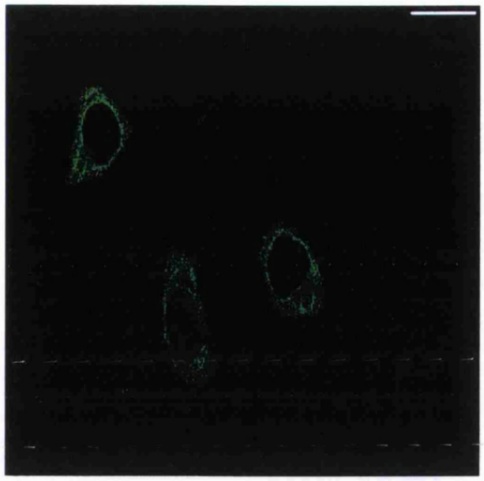


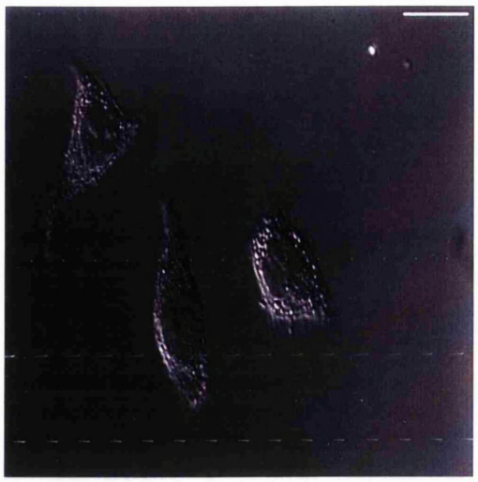
***Fig.6.8**: **Distribution of XOR in permeabilised EA.hy926 cells**.*
Affinity purified rabbit polyclonal anti-HXOR (0.026mg/ml) and anti-rabbit FITC conjugated (1:100) antibodies were used to localise XOR inside the cells. Immunofluorescent (A, C, E) and differential interference contrast (DIC) (B, D, F) images are shown. Magnification x600; laser emission confocal fluorescence cytometer.

"Permeabilised EA.hy 926 cells":

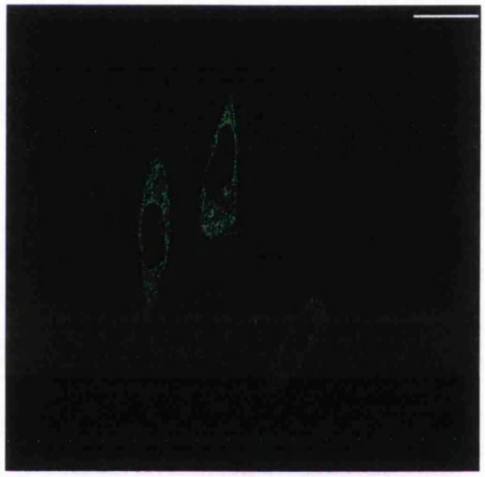
***Fig.6.9*: Distribution of XOR in permeabilised EA.hy 926 cells.**
Cells were prepared as described in Section 2.7. Immunofluorescent (A, C, E) and DIC (B, D, F) images are shown. Antibodies used: affinity purified rabbit polyclonal anti-HXOR antibody (stock: 0.1mg/ml, 1:2 dilution was used) and anti-rabbit FITC conjugated antibody (1:100). Magnification x630 (A-D), x400 (E-F); 20μm bar.



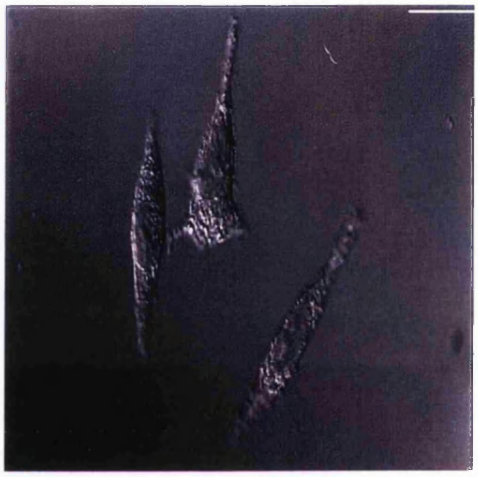
A



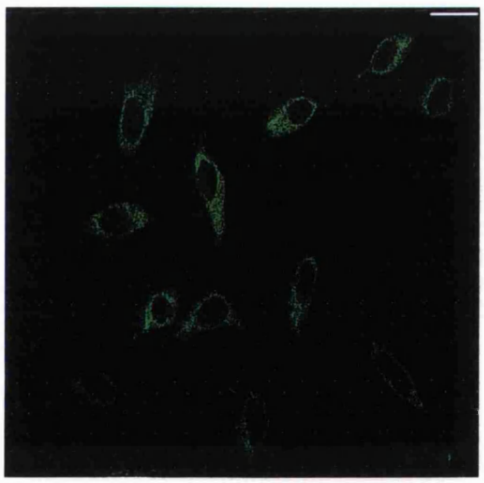
B



C



D



E

F

Permeabilised EA.hy 926 cells.

The perinuclear membrane consists of two unit membranes enclosing a perinuclear space. In many protein secreting cells, the outer membrane is identical to rough endoplasmic reticulum, with attached ribosomes and a perinuclear space swollen with protein secretions (Cross and Mercer, 1993). The localisation of an enzyme capable of producing ROS in the cytoplasm of cells, and more precisely in the perinuclear region of the cells, has interesting implications for the function of the enzyme and will be examined in the discussion.

6.3.3.2. Localisation of XOR on the outer surface of human endothelial cells

Fig.6.10 shows the presence of XOR on the surface of cells using a confocal fluorescence cytometer (Section 2.7.1). For this purpose, mouse monoclonal anti-HXOR antibodies were used. The antibodies are seen to be concentrated at the junction of the cells. Immunolocalisation of XOR in unpermeabilised cells with a confocal laser-scanning microscope (Fig.6.11) also showed the presence of the enzyme on the outer surface. Moreover, XOR appeared to be concentrated on parts of the surface that apposed or were extending towards neighbouring cells (arrows on Fig.6.11).

A fluorescent microscope was also used to verify the distribution of XOR enzyme on the surface of EA.hy 926 cells (Fig.6.12). Similar results to those with the confocal microscope were obtained. Monoclonal and affinity purified antibodies gave similar results, the latter being more punctate.

6.3.3.3. Control experiments

It is clear that the subcellular localisation of XOR is very well defined. Much of the enzyme is in the perinuclear space and significant amounts of the protein can be seen to be extracellular. In the latter case, strict controls must be set up because the 'cross reacting material' may not be exogenous cellular XOR. It is quite likely that the serum in the medium contains soluble XOR which itself might become non-specifically bound to the outside surface of the cells. This is a crucial problem and several steps were taken to show that the medium could not have contained any XOR for cell binding. The first approach was to assay total calf serum for XOR activity by fluorescent assay (Section 2.3.2). No detectable XOR was seen.

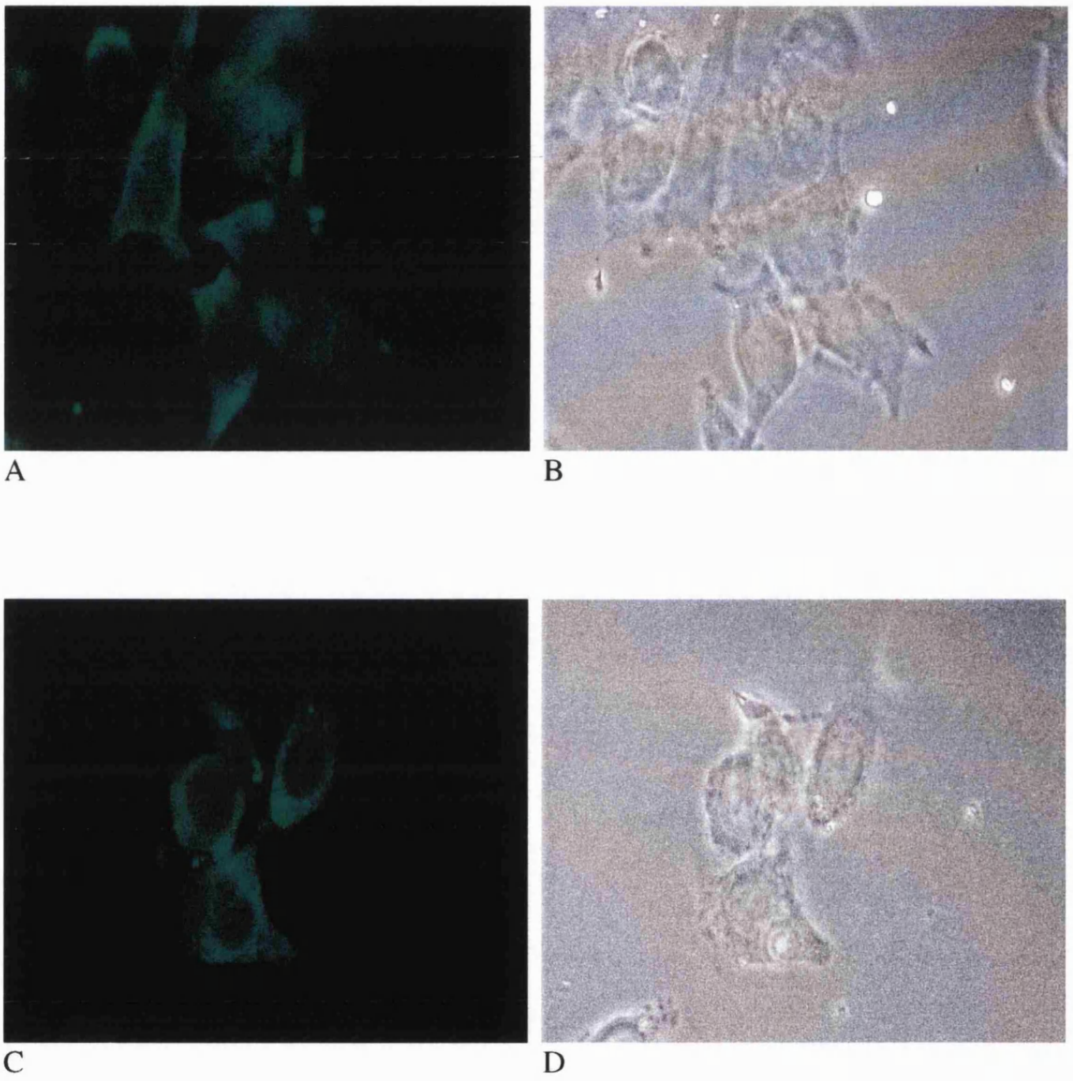


***Fig.6.10*: Distribution of XOR in unpermeabilised EA.hy 926 cells.**
Mouse monoclonal anti-bovine XOR (50μl) and anti-mouse FITC conjugated (1:100) antibodies were used to localise XOR on the outer surface of the cells. Immunofluorescent (A, C) and DIC (B, D) images are shown. Magnification x600; laser emission confocal fluorescence cytometer.

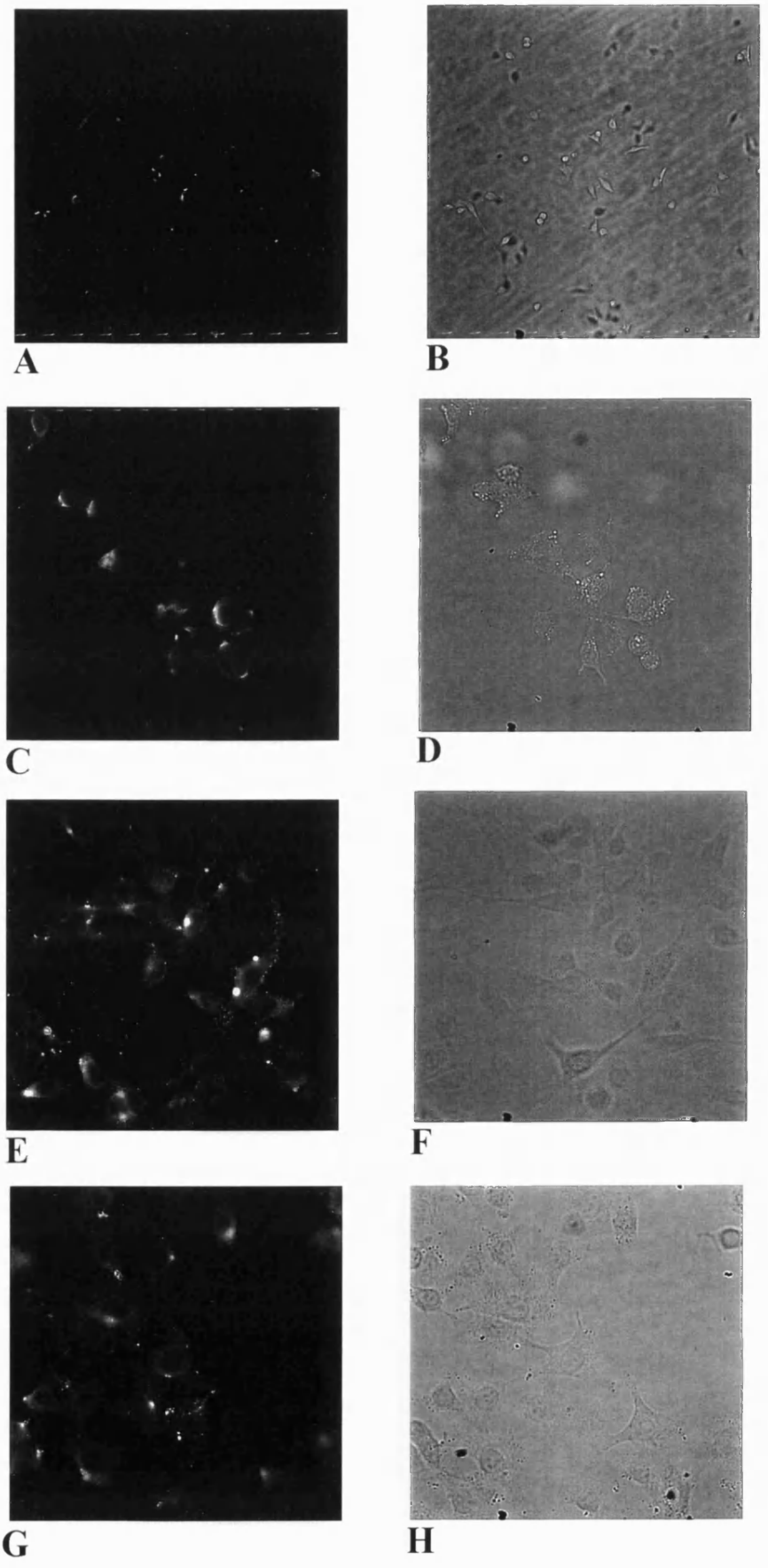
"Unpermeabilised EA.hy 926 cells":

***Fig.6.11*: Distribution of XOR in unpermeabilised EA.hy 926 cells.**
Cells were prepared as described in Section 2.7. Immunofluorescent (A, D, G) and DIC (B, E, H) images are overlaid (C, F, I) to emphasise the polarised distribution. Arrows (C) show examples where fluorescence is concentrated on surfaces that appose those of neighbouring cells. Antibodies used: affinity purified rabbit polyclonal anti-HXOR antibody (stock: 0.1mg/ml, 1:2 dilution was used for D-F. Stock 0.221mg/ml, 1:10 dilution was used for A-C and G-I) and anti-rabbit FITC conjugated antibody (1:100). Magnification x400 (A-C, G-I), x630 (D-F); 20μm bar.

Unpermeabilised EA.hy 926 cells.

"Unpermeabilised EA.hy 926 cells-Fluorescent microscope":

***Fig.6.12*: Distribution of XOR in unpermeabilised EA.hy 926 cells, using a fluorescent microscope.**
Cells were prepared as mentioned in Section 2.7. Immunofluorescent (A, C, E, G) and DIC (B, D, F, H) images are shown. Antibodies used: mouse monoclonal anti-HXOR antibody (4.3mg/ml IgG from clone 8) used to a final concentration of 0.15mg/ml for A-D or affinity purified rabbit polyclonal anti-HXOR (stock: 0.55mg/ml, 1:13 dilution was used) for E-H and anti-rabbit FITC conjugated antibody (1:100). Magnification x 400.



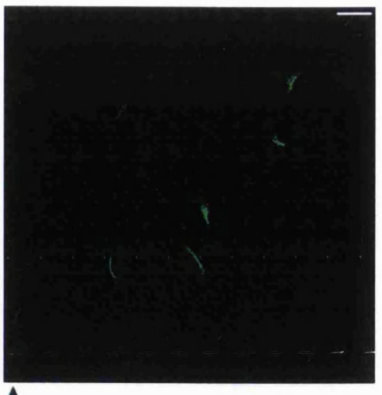
Unpermeabilised EA.hy 926 cells-Fluorescent microscope.

In a second control experiment, any XOR present but undetectable in the serum used to make the medium was removed using affinity chromatography (Section 2.8), and this XOR-depleted serum was then used to perform the fluorescent localisation of XOR (Section 2.7.3). XOR has high affinity for heparin (Adachi *et al*, 1993; Sanders *et al*, 1997), and heparin-agarose columns were used to remove any XOR present in the medium (Section 2.8). Different batches of serum (six in total) were run through the heparin columns; the columns were washed with 25mM sodium phosphate buffer and then eluted with 1M NaCl and the eluants were tested for XOR activity by fluorescence assay. In fact, no activity was detectable in these different eluants. The use of serum which was known to be free of XOR was considered to be an unambiguous proof that the extracellular bound XOR was in fact truly extracellular and not derived from the medium. Cells were grown in medium which had been passed down heparin-agarose. The cells were set as usual in glass wells and were subjected to immunolabelling at the same time as cells grown in normal medium. XOR was seen to be localised in both cases on the surface of the cells (Fig.6.13, A-D) and there was no observable difference between the two groups of cells. When assayed on the fluorimeter, no significant difference was apparent in the total XOR expressed in the two groups of cells.

In a further control experiment, cells were set and treated as usual, except that FITC conjugated secondary antibody was omitted and replaced by PBS. No fluorescence was observed. Secondly, the primary antibody was omitted and only the secondary antibody FITC was added to the cells. Again, no fluorescence was observed (Fig.6.13, E-H). These control experiments verify the specificity of the antibodies used for the distribution of XOR. It allows to check first that there is no background of fluorescence and secondly that the secondary antibody only binds to the primary one. It can be noted that commercially supplied antibody, rabbit anti-bovine milk XOR (Chemicon International), gave similar results with permeabilised and unpermeabilised EA.hy 926 cells to those obtained by using locally produced antibody.

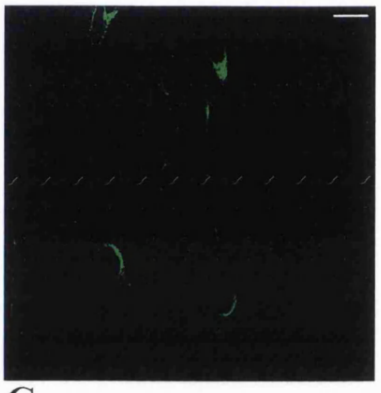
"Controls":

***Fig.6.13*: Control experiments testing antibodies and heparin-Agarose treatment of medium.**
EA.hy 926 cells were grown in medium that had (C, D) or had not (A, B) been preabsorbed on a heparin-Agarose column (Section 2.8). For the antibody controls, cells were treated as usual except that antibodies were replaced by PBS: secondary antibody (E, F), primary antibody (G, H). Immunofluorescent (A, C, E, G) and DIC (B, D, F, H) pictures were obtained as described in Section 2.7. Antibodies: affinity purified rabbit polyclonal anti-HXOR antibody (Stock: 0.221mg/ml, 1:10 dilution was used for A-D. Stock: 0.54mg/ml, 1:10 dilution was used for E-H) and anti-rabbit FITC conjugated antibody (1:100). Magnification x400; 20μm bar.



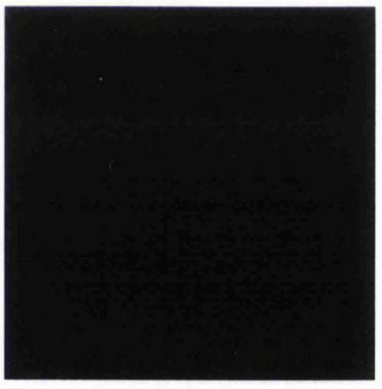
A

B



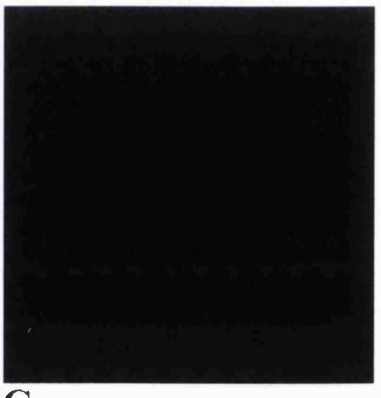
C

D

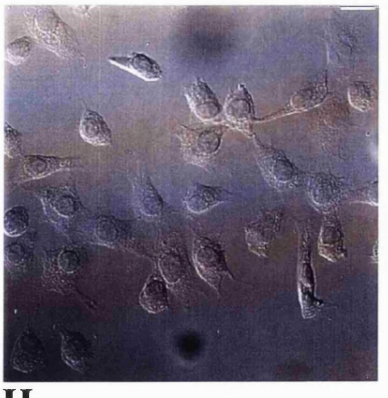


E

F



G



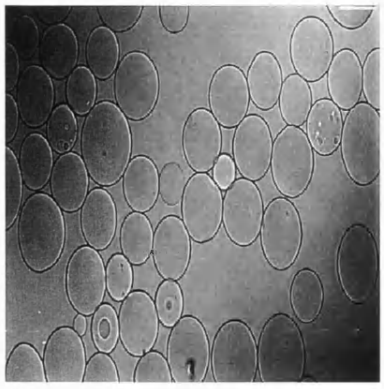
H

Controls.

"Heparin beads":

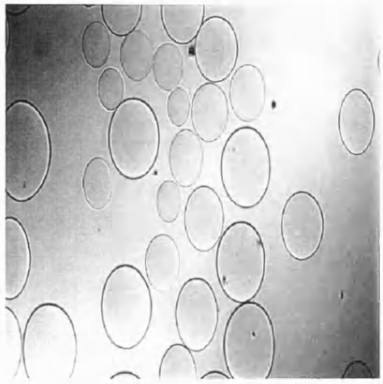
***Fig.6.14:* Control experiments showing that cell surface XOR is not derived from growth medium.**
Heparin beads were incubated for 24h with PBS (A-B), with PBS containing bovine XOR (10μg/ml) (C-D), with NGS (E-F) and with growth medium (G-H). Immunofluorescent (A, C, E, G) and DIC (B, D, F) images were obtained as described in Section 2.7. Antibody: affinity purified rabbit polyclonal anti-HXOR antibody (stock: 0.5mg/ml, 1:20 dilution was used) and anti-rabbit FITC conjugated antibody (1:100). Magnification x100, 50μm bar.

A



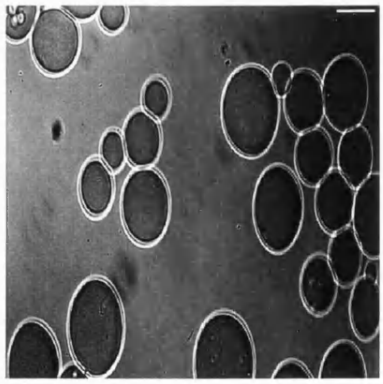
B

C

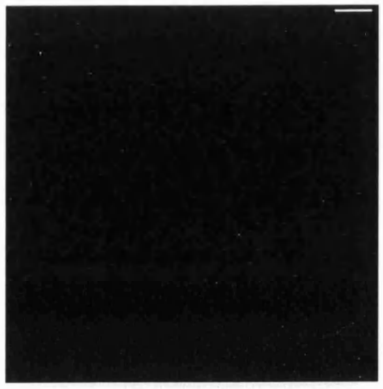


D

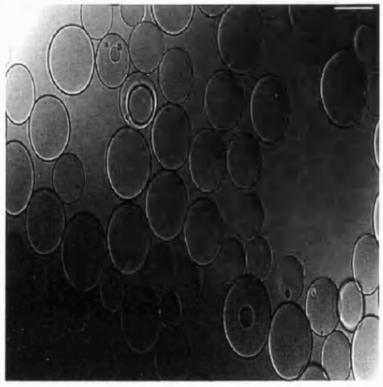
E



F



G



H

Heparin beads.

In a final control experiment, heparin beads (300µl) were first washed with PBS before being incubated overnight with either PBS, bovine XOR (10µg/ml), NGS (normal goat serum) or growth medium. The beads were then labelled with affinity purified rabbit polyclonal anti-HXOR antibody (Section 2.7). Clear fluorescence was observed on beads incubated with bovine XOR (Fig.6.14, C-D). No fluorescence was found in the case of PBS, NGS or pure growth medium. NGS was tested as it was added to the antibody at the same time as BSA, as a blocking agent (Fig.6.14, A-B and E-H)

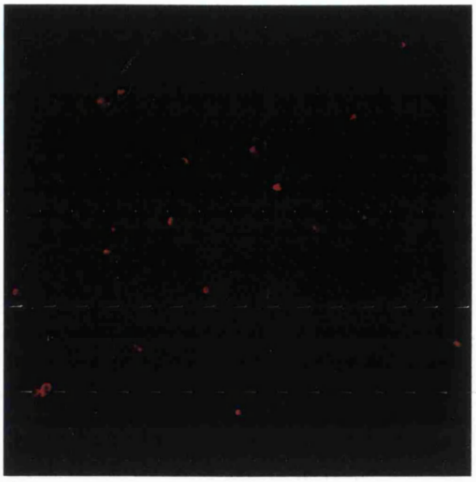
6.3.3.4. Subcellular localisation of *trans* Golgi marker, TGN38

Anti-TGN38, a mouse anti-*trans* Golgi antibody, was used as a further control to show that cells were permeabilised and also to specify the localisation of XOR inside the cells. TGN38 was originally identified as a marker for the *trans*-Golgi network in the mouse (TGN) (Luzio *et al*, 1990), and the antibody was shown to cross-react with human antigen (Ponnambalam *et al*, 1996). TGN38 used here as an intracellular marker protein was not stained by anti-TGN38 antibody in unpermeabilised cells (Fig.6.15, C-D). However, cytoplasmic staining was seen in permeabilised cells (Fig.6.15, A-B). This result confirms preliminary experiments in which the status of permeabilised and unpermeabilised cells was verified by staining with trypan blue. Permeabilised and unpermeabilised cells were stained with trypan blue (500µl/well) for 5min, then three washes with PBS (1ml/wash) were carried out. The staining of the cells was checked under a Nikon microscope, magnification x400. It was found that permeabilised cells took up 100% of the trypan blue compared with unpermeabilised cells, which took up only 5%.

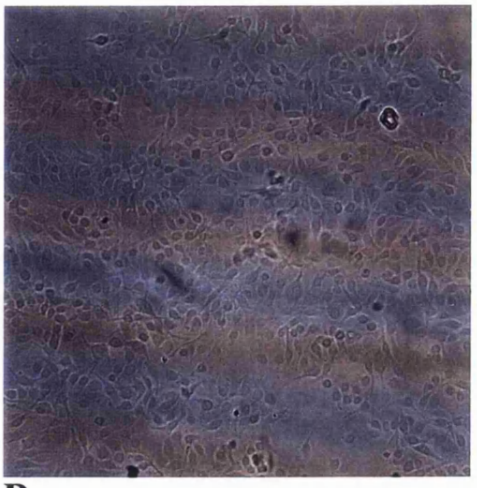
Colocalisation of XOR and TGN38 (XOR with FITC conjugated antibody and anti-TGN38 with rhodamine linked antibody), was then examined. Permeabilised cells were first fixed and immunolocalisation of XOR was carried out with affinity purified rabbit polyclonal and anti-rabbit FITC conjugated antibodies. Then colocalisation was carried out with mouse anti-TGN38 and anti-mouse rhodamine conjugated antibodies (Section 2.7). In permeabilised cells, TGN38 and XOR were colocalised in most of the cytoplasm (Fig.6.15, E-F), apart from the perinuclear region which showed only XOR.

"TGN38":

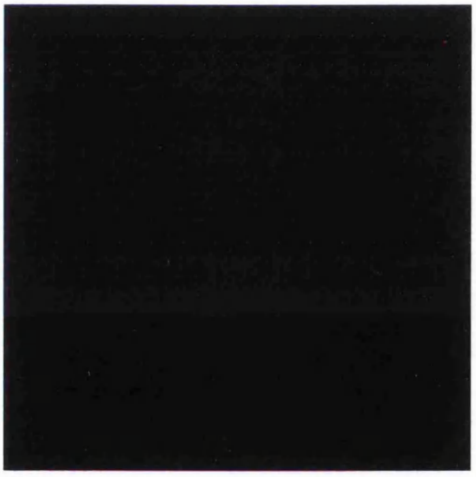
***Fig.6.15*: Distribution of TGN38 in permeabilised (A, B, E, F) and unpermeabilised cells (C-D).**
Cells were prepared as described in Section 2.7. Immunofluorescence (A, C, E) and DIC (B, D, F) images are shown. Antibodies: mouse anti-TGN38 antibody (1:100) for A, C, E, together with affinity purified rabbit polyclonal anti-HXOR (Stock: 0.5mg/ml, 1:20 dilution was used) for E and anti-rabbit FITC conjugated antibody (1:100) for A, C, E as well as anti-mouse rhodamine conjugated antibody (1:100) for E. Magnification x 100 (A-D. Fluorescent microscope), x400 (E, F. Confocal microscope); 20μm bar.



A



B



C

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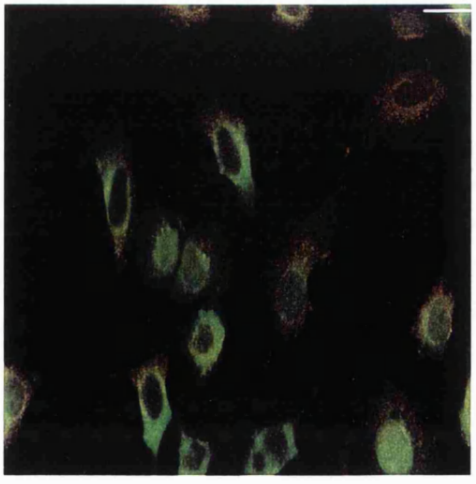


E

F

TGN38.

6.3.3.5. Localisation of free radicals using dihydrorhodamine 123

Dihydrorhodamine 123 (DHR123) is oxidised to rhodamine 123 (R123), a fluorescent product, and is used as a specific fluorescent probe for visualising mitochondria in living cells (Sobreira *et al*, 1996). DHR123 can be oxidised by hydrogen peroxide in the presence of a cellular enzyme with peroxidase activity (Rothe and Valet, 1990; Henderson and Chappell, 1993), and is considered to be a good sensor for ROS (Vowells *et al*, 1995; Yvonne *et al*, 1995; López-Ongil *et al*, 1998).

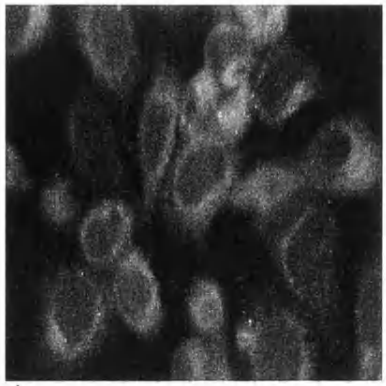
DHR123 was directly added to the medium and incubated with the cells for 45min at 37°C. Cells were previously treated with a final concentration of 50μM allopurinol for 24h. After 45 min, cells were washed three times with prewarmed PBS, fixed and mounted as described in Section 2.7. The presence of ROS was compared in cells treated with allopurinol, which had been shown to reduce XOR activity in EA.hy 926 cells (Chapter 5), and in non-treated cells. As shown on Fig.6.16, cells treated with allopurinol for 24h have less staining than control cells. Moreover, the pattern of the staining is more punctate and concentrated to a few points which may correspond to the mitochondria. In the controls, ROS seem to be dispersed though the cytoplasm of the cells.

6.3.3.6 HUVECs

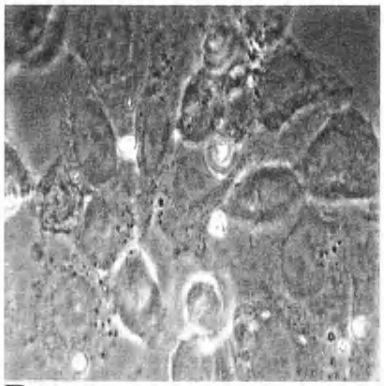
The experiments described with EA.hy 926 cells were repeated with HUVECs. HUVECs, isolated from human umbilical veins, were grown in 25cm^2 flasks. When confluent, the cells were split and seeded in four well chambers. The same conditions as those described for EA.hy 926 cells were used (Section 2.7). Again, it was found that the enzyme was present in the cytoplasm of the primary cells, and around the perinuclear region of permeabilised cells. XOR was also present on the surface of the cells (Fig.6.17). The specific distribution of XOR in the perinuclear region and on the outer surface of the cells is accordingly as specific as in EA.hy 926 cells. No direct comparison on the fluorescence intensity was carried out; however, it is clear that the enzyme is present in the cells, even if no enzymatic activity was detectable by fluorimetric assay.

"DHR123":

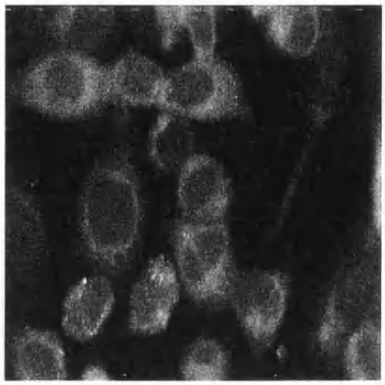
***Fig.6.16*: Distribution of free radicals in EA.hy 926 cells.**
Immunofluorescent (A, C, E, G) and DIC (B, D, F, H) images are shown. On day 0, cells were seeded at $3x10^5$ cells/ml. On day 1, allopurinol was added to E-H to a final concentration of 50μM per well. On day 2, DHR123 was added to all the samples to a final concentration of 24μM, for 45min at 37°C. The cells were then washed and fixed with 4% formaldehyde. Finally, the cells were washed with PBS and mounted. Magnification x600, laser emission confocal fluorescent cytometer.



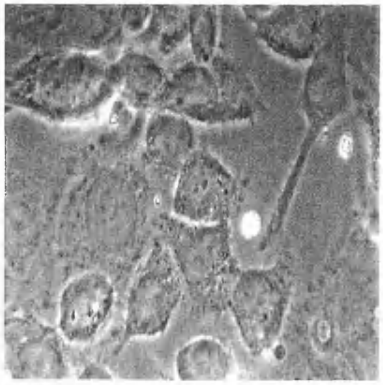
A



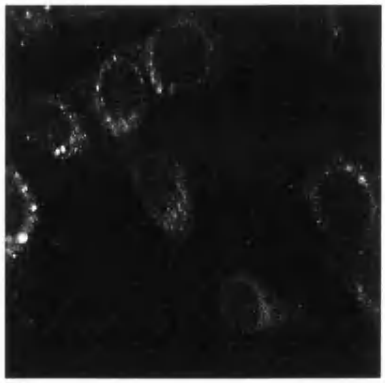
B



C



D



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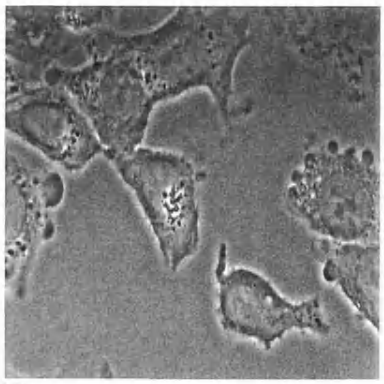
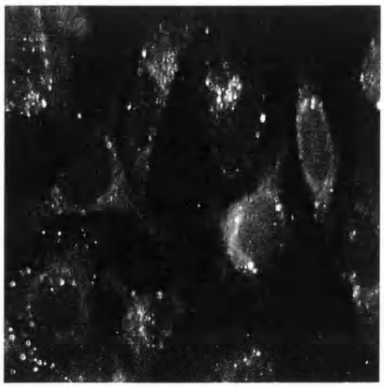
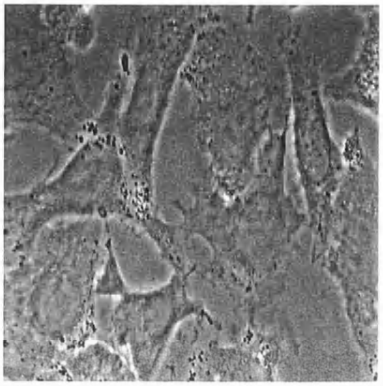


F



G

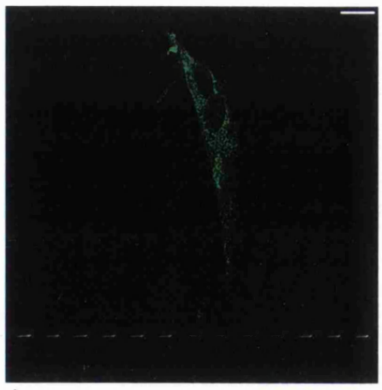


H

DHR123.

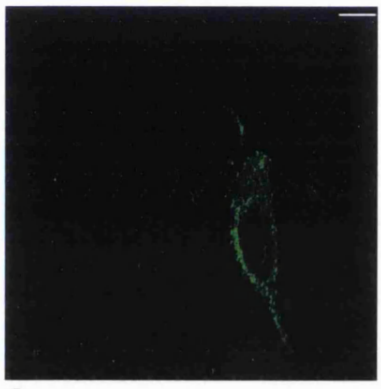
"HUVECs":

***Fig.6.17*: Distribution of XOR in permeabilised (A-B) and unpermeabilised (C-G) HUVECs.**
Cells were prepared as described in Section 2.7. Immunofluorescent (A, C, E) and DIC (B, D, F) images are shown. Images E and F are overlaid (G) to emphasise the polarised distribution. Antibodies used: affinity purified rabbit polyclonal anti-HXOR (stock: 0.221mg/ml, 1:10 dilution was used for C-G. Stock: 0.175mg/ml, 1:10 dilution was used for A-B, F-H) and anti-rabbit FITC conjugated antibody (1:100). Magnification x400; 20μm bar.

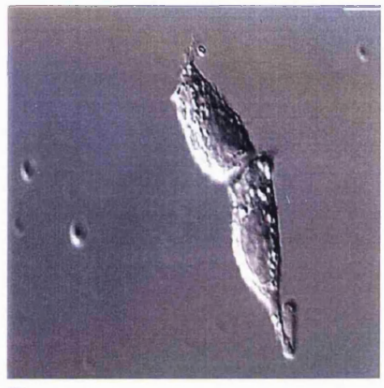


A

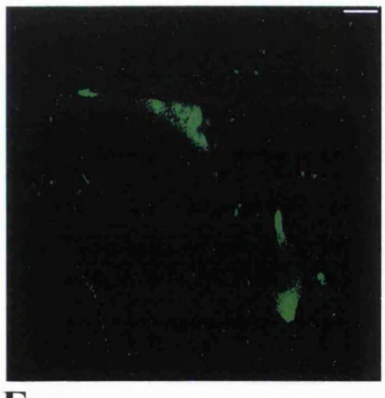
B



C

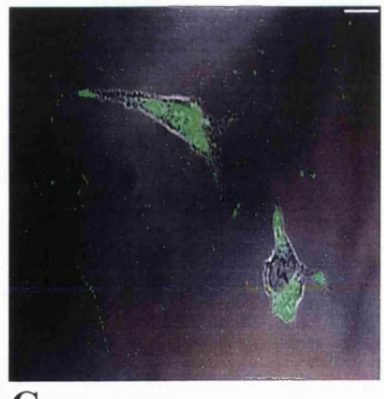


D



E

F



G

HUVECs.

6.4. Conclusions

A 150 kDa band corresponding to XOR protein was clearly seen following SDS-PAGE and Western blotting of an immunoprecipitate from the cytoplasmic supernatant of EA.hy 926 cells. Western blots on crude samples were positive but not as clear as for those obtained by immunoprecipitation. This can be due to the loss of protein during the transfer and the fact that the experiments were carried out on the cell supernatant, i.e. on a very low quantity of proteins. The specificity of the antibodies was additionally tested with HXOR purified from human milk. These antibodies were then used for the specific localisation of XOR inside and outside different human cell lines as described in the following paragraphs.

The anti-HXOR antibodies, verified as above, were used to clarify the distribution of XOR in EA.hy 926 cells as well as in HUVECs. XOR enzyme was found in the cytoplasm of the cells as expected, but particularly concentrated in the perinuclear region. This may well reflect its proposed function. As previously mentioned, ROS were shown to activate some transcription factors, such as nuclear factor NF-κB (Schreck *et al*, 1991; Meyer *et al*, 1993; Sen and Packer, 1996). XOR is a major source of ROS, and the presence of XOR might be linked to the presence of inactive complex I-κB and NF-κB in the cytoplasm (see General Introduction).

Another finding was that the presence of ROS inside the cells can be reduced and modified by the treatment of the cells with allopurinol for 24h. The presence of XOR on the outer surface of endothelial cells has not been previously published, apart from brief abstracts reporting the extracellular localisation of XOR in bovine aortic endothelial cells (Bulkley, 1991; Schiller *et al*, 1991). This finding presence raised questions about the function of the enzyme. The main question is whether the enzyme is active and this idea will be developed in Chapter 8. To verify that these results were not an artefact, different controls were carried out. It was first shown that similar results were obtained using a fluorescent microscope as well as commercial antibody. It was then checked that the cells were really not permeabilised by the method used. For this an internal marker (TGN38) was used and the results were compared between permeabilised and unpermeabilised cells.

The last control consisted of showing that XOR present on the outer surface of unpermeabilised cells could not be due to the presence of XOR in the medium and particularly in the serum. Cells were grown in medium in which XOR was removed using an heparin column. These cells still showed presence of XOR on their surface. Different compounds used in the methods were also tested for the possible presence of XOR, but the results were negative.

It can be noted that a similar distribution of XOR in the cytoplasm and on the outer surface of cells was found in mammary epithelial cells, HB4a (Rouquette *et al*, 1998) and also in mouse fibroblast cells, L929 (Chapter 7).

CHAPTER 7
THE L929 CELL LINE.

7.1. Introduction

L929, a mouse fibroblast cell line, was one of the first cell lines to be established in continuous culture (Earle *et al*, 1943). In the present context, L929 cells are unique because of their capacity to show XOR enzymic activity only in presence of molybdenum (IV) salts. In 1994, Falciani and coworkers demonstrated that L929 cells show no detectable XOR activity either under normal conditions or following stimulation with cytokines. However, when a molybdenum salt was added to the cells, significant levels of XOR activity were detected under all conditions. This induction was thought to be post-translational as it was insensitive to cycloheximide, indicating that inactive enzyme protein was present in cells prior to addition of molybdenum.

In this Chapter, the L929 cell line was used as another approach to study the regulation of XOR expression. The results of Falciani *et al* (1994) were checked before investigating whether similar effects could be observed with EA.hy 926 cells. The distribution of XOR was studied in permeabilised and unpermeabilised L929 cells both before and after exposure to molybdenum, in order to compare results with those obtained in human endothelial cells (Chapter 6).

7.2. Methods

L929 cells were cultured as described in Section 2.1.2.2. On day 0, the cells were seeded at $2x10^5$ cells/ml. For studies of molybdenum addition, sodium molybdate was added to the medium of L929 cells or EA.hy 926 cells to a final concentration of 0.5mM, from day 0 and every time the medium was changed. Cell supernatants were prepared as described in Section 2.1.4, and fluorimetric assays were carried out as in Section 2.3. The fluorimetric assay was slightly modified from that of Section 2.3 in that XOR activity was measured in the absence and presence of methylene blue, in order to determine XO and XO + XDH activities respectively. The distribution of XOR was analysed in permeabilised and unpermeabilised cells following the procedures described in Section 2.7.

7.3. Results

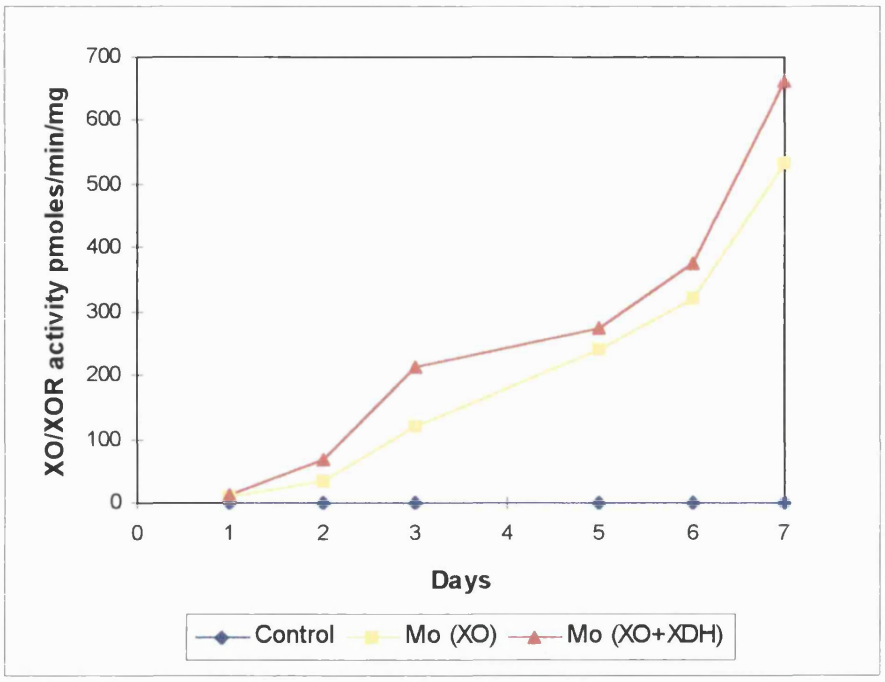
7.3.1. Effects of molybdate on expression of XOR enzymic activities

In the absence of molybdate, L929 cells expressed no detectable XOR activity. When molybdate salt was added to the medium, XOR activity began to be detectable after only 24h. Activity continued to increase with days in culture as shown in Fig.7.1 A. The controls (no addition of molybdate) showed no detectable XOR specific activity (Fig.7.1 A). XO activity represented 60% to 80% of the total XOR activity (XO + XDH). Molybdate added to the EA.hy 926 cell line did not induce any significant increase in XOR activity (Fig.7.1, B) compared to the controls.

7.3.2. Distribution of XOR in L929 treated or not with molybdate salt

The distribution of XOR was investigated by means of confocal microscopy and immunolocalisation using affinity-purified anti-HXOR antibody. Quantitative comparisons between cells treated with added molybdate and non-treated cells were carried out by using the same settings on the confocal microscope.

A)



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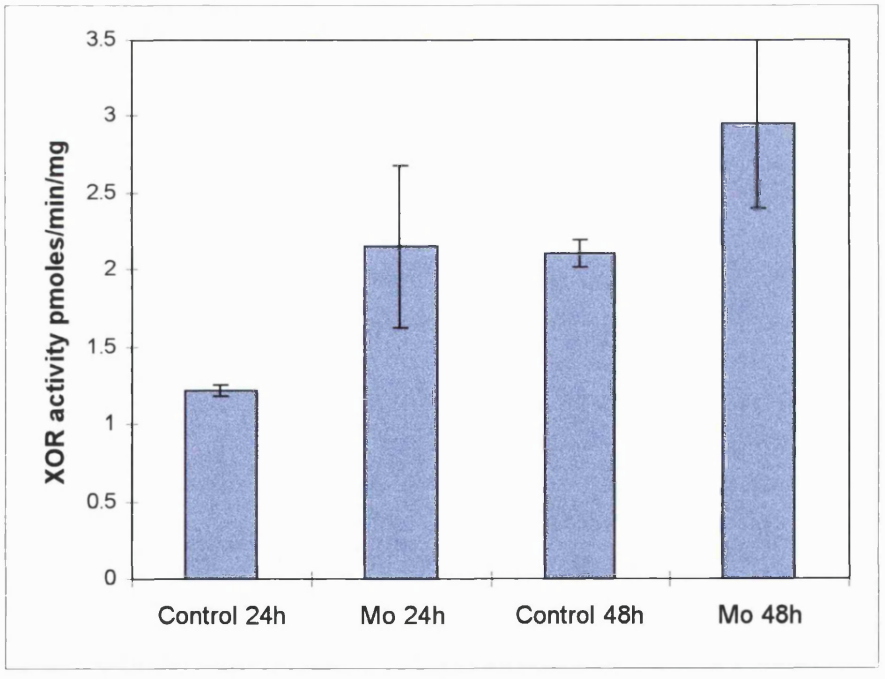


***Fig. 7.1*: Effects of molybdate on L929 and EA.hy 926 cells.**
A final concentration of 0.5mM molybdate (Mo) was added to the cells. A) L929 cell line, XO and XO+XDH activities are shown. B) EA.hy 926 cell line, XO+XDH activity only. Values are quoted as mean ± SEM; n=2.

7.3.2.1. Permeabilised L929 cells

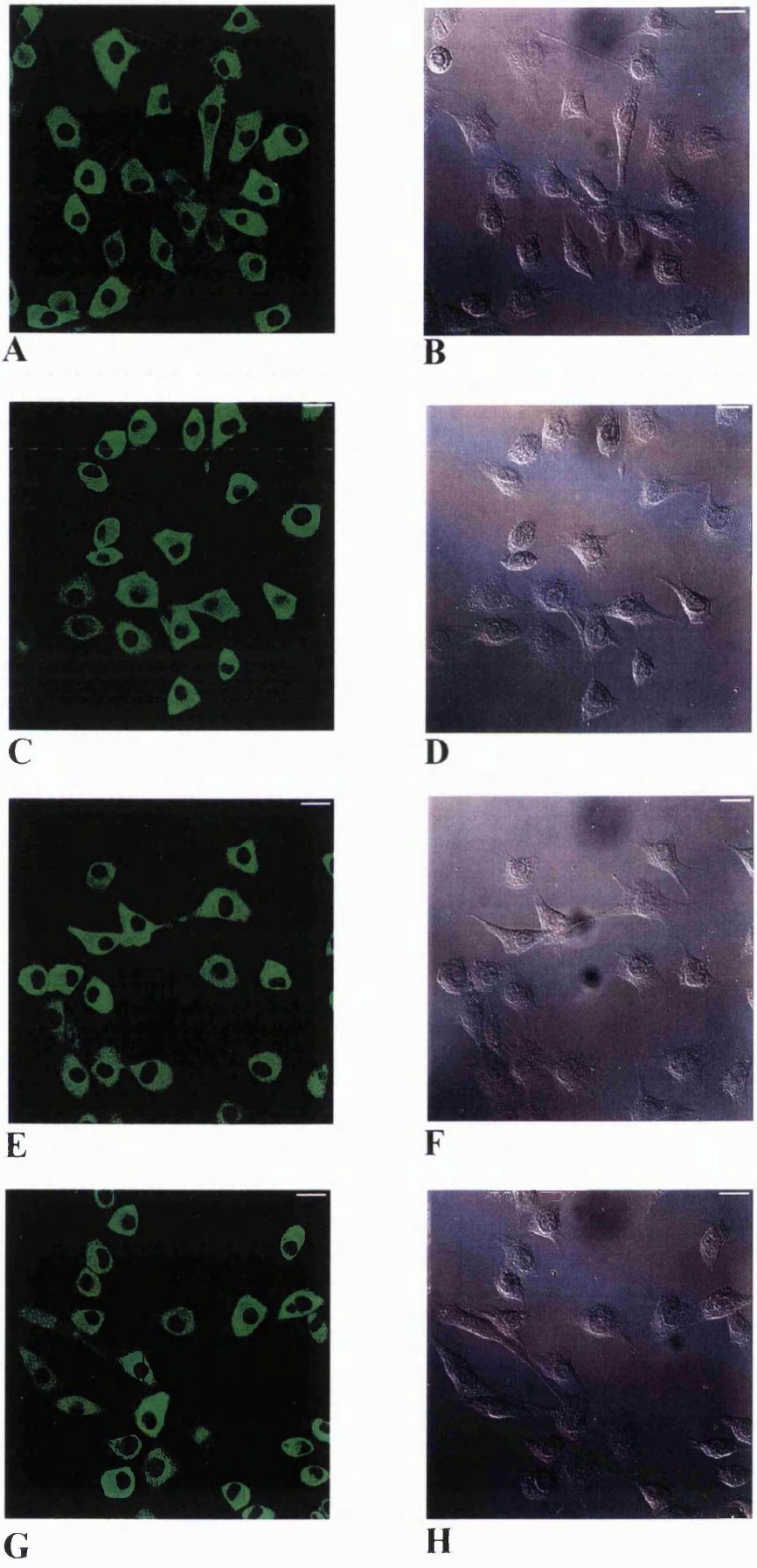
As in EA.hy 926 cells, XOR was found to be present in the cytoplasm of L929 cells with increased fluorescence around the perinuclear region. The cells treated with molybdate initially appeared brighter than controls, and so the confocal microscope conditions were set up to the cells treated with molybdate (Pictures A-D), and images were collected from the controls under similar conditions (Pictures E-F). When the two groups were compared, no significant difference was apparent. However, fluorescence was clearly stronger in L929 cells (Fig.7.2) than in EA.hy 926 cells (Fig.6.9), although quantitative comparisons were not possible.

7.3.2.2. Unpermeabilised L929 cells

Again, results for L929 cells were similar to those found for EA.hy 926 cells or HUVECs. XOR protein was seen to be present on the outer surface of the cells, whether or not they were grown in medium containing 0.5mM molybdate. Photographs of cells treated with molybdate and controls were collected under similar conditions on the confocal microscope, and no significant difference was found between the two groups. However, the pictures do suggest that the presence of XOR enzyme on the surface of the cells is more striking in the case of the L929 cell line (Fig.7.3) than in the case of EA.hy 926 cells (Fig.6.11), although no quantitative comparisons were possible. The presence of XOR is very polarised on the surface of L929 cells. For most of the cells, XOR is only present on one side of them. The arrows on Fig.7.3, indicate the junctions of cells where XOR is usually present. Overlaid fluorescence and DIC pictures (S, F, I, L) emphasise the relationship between presence of the enzyme and junction of two or more cells.

"Permeabilised L929 cells":

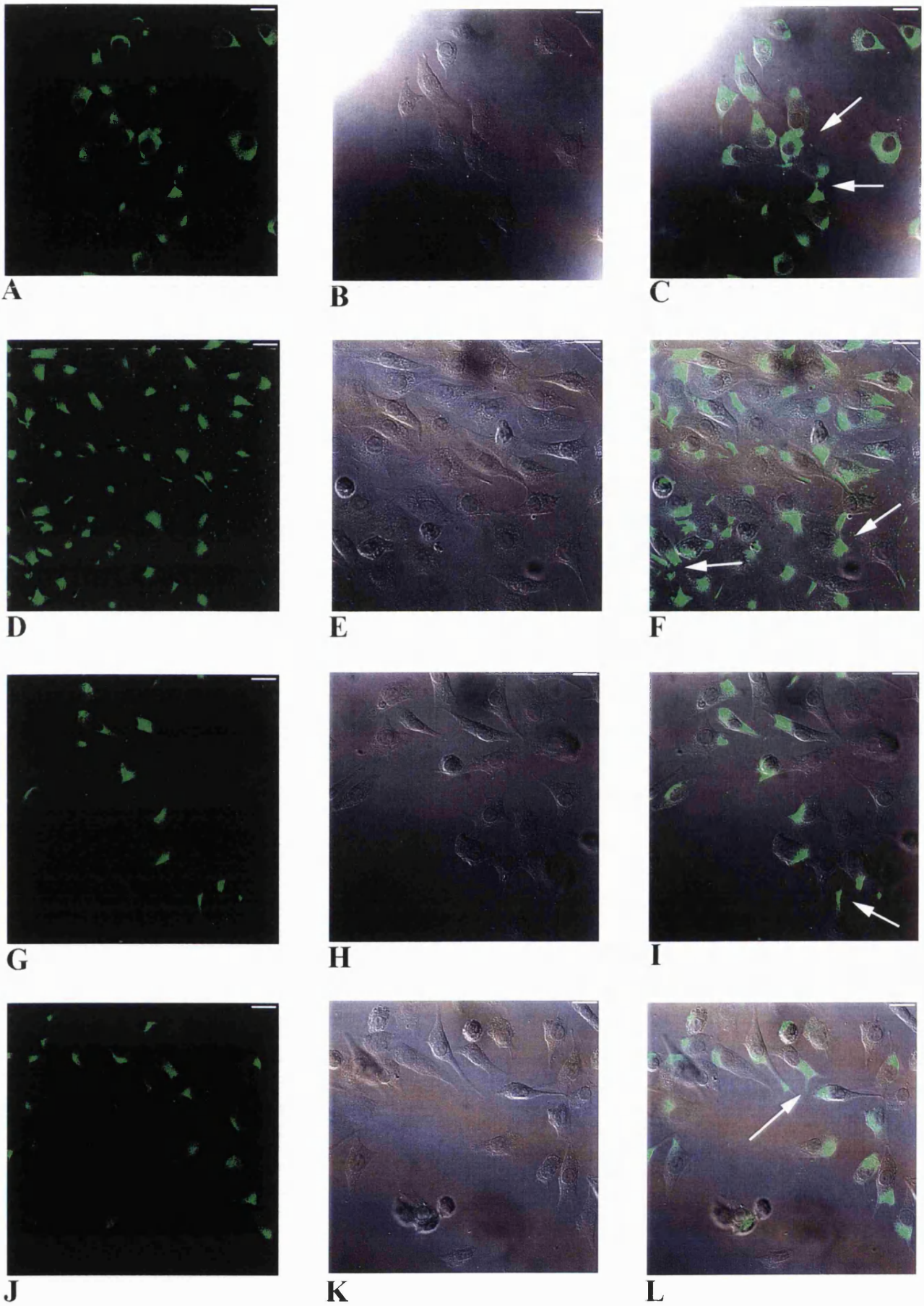
***Fig. 7.2*: Distribution of XOR in permeabilised L929 cells.**
Cells were prepared as mentioned in Section 2.7. Immunofluorescent (A, C, E, G) and differential interference contrast (DIC) (B, D, F, H) images are shown. Cells were grown either in medium containing molybdate (A-D) or in normal medium (E-F), see Section 2.1.2.2. Antibodies used: affinity purified anti-HXOR antibody (stock 0.54mg/ml, 1:10 dilution was used) and anti-rabbit FITC conjugated antibody (1:100). Magnification x400; 20μm bar.



Permeabilised L929 cells.

"Unpermeabilised L929 cells":

***Fig. 7.3*: Distribution of XOR in unpermeabilised L929 cells.**
Cells were prepared as mentioned in Section 2.7. Immunofluorescent (A, D, G, J) and DIC (B, E, H, K) images are overlaid (C, F, I, L) to emphasise the polarised distribution. Cells were grown either in medium containing molybdate (A-F) or in normal medium (G-L), see Section 2.1.2.2. Antibodies used: affinity purified anti-HXOR antibody (stock 0.54mg/ml, 1:10 dilution was used) and anti-rabbit FITC conjugated antibody (1:100). Magnification x400; 20μm bar.



Unpermeabilised L929 cells.

7.4. Conclusions

According to Falciani *et al* (1994), activation of XOR by molybdenum (IV) salts was specific to the L929 cell line, and in my work, this was not observed in EA.hy 926 cells. Studying L929 cells provides another model for the expression of XOR in cell culture. The fact that, after many years of culture, XOR is still expressed in these cells in an inactive form, lacking Mo and/or its cofactor, suggests that the XOR protein may play a more complicated role in the cell than is apparent.

EA.hy 926 and L929 cells must have access to a similar amount of molybdenum initially present in the medium, in normal conditions. However, the L929 cells are incapable of utilising directly this source of molybdenum salt. Exogenous molybdenum salt must be added to the medium in a relatively high quantity, for XOR to be fully active. This raises the question of which pathway the exogenous molybdenum uses to activate XOR.

Molybdoenzymes have been particularly well studied in *E.coli,* in which the production of the enzyme relies upon the efficient uptake of molybdate via the molybdate specific transporter encoded by the *mod*ABCD operon (Grunden *et al*, 1996). Rajagopalan and Johnson (1992) showed that some *E.coli* mutants, referred as '*mod*' and '*mog*' were unable to synthesise the molybdenum cofactor essential for these molybdoenzymes. However, both mutants have been reported to respond to elevated levels of molybdate in the culture medium (Rajagopalan and Johnson, 1992; Shanmugam *et al*, 1992). Joshi and colleagues (1996) proposed that molybdopterin might be synthesised normally but might be subjected to degradation if further assembly into molybdenum cofactor and incorporation into molybdoenzymes was interrupted, leaving low levels of residual pterin in the cells. On the other hand, the mutant cells respond to elevated levels of the metal in culture because molybdate uptake can be accomplished by mass action through transport by cellular phosphate or sulphate uptake systems.
This system was described in *E.coli* and more studies need to be carried out on the subject on the mammalian enzyme.

It is also conceivable that post-translational activation of XOR could be by way of molybdenum incorporation into basically depleted cells (Godber *et al*, 1997). However, as mentioned before, EA.hy 926 cells and HB4a cells are capable of using molybdenum salt present in their respective media to express an active XOR enzyme. L929 cells need an excess of molybdate in their medium for XOR to be active. The system of incorporation of molybdenum salt into the cells might not be efficient in L929. No increase in XOR activity was observed in EA.hy 926 cells incubated with 0.5mM molybdate. The result of this experiment does not preclude the existence of some enzyme-driven regulatory mechanism involving molybdenum incorporation.

CHAPTER 8
DETERMINATION OF NADH OXIDASE ACTIVITY ON THE OUTER SURFACES OF CELLS.

8.1. Introduction

XOR commonly exists *in vivo* as the xanthine dehydrogenase (XDH) form which utilises NAD^+ as an electron acceptor during the oxidation of xanthine. Xanthine oxidase (XO), on the other hand, utilises molecular oxygen as its electron acceptor, giving rise to ROS (Hille and Massey, 1991; Nishino and Hille, 1995). A little recognised activity of XOR is its ability to act as an NADH oxidase. The NADH oxidase activities of the dehydrogenase and the oxidase forms of the human XOR were studied by Sanders *et al* (1997). It was found that the human milk enzyme was capable of oxidising NADH to form superoxide anions, particularly in the case of the dehydrogenase form. NADH differs from other reducing substrates in acting at the FAD, rather than the Mo site. No specific inhibitor for the FAD centre of XOR has been reported, while diphenyleneiodonium chloride (DPI) will certainly inhibit this activity. Several flavoproteins are similarly inhibited, including NADPH oxidase of phagocytes (Cross and Jones, 1986; O`Donnell *et al*, 1993), nitric oxide synthase (Stuehr *et al*, 1991) and NADPH-cytochrome P-450 reductase (Doussière and Vignais, 1992). Allopurinol inhibits only the molybdenum centre of XOR and does not interact with the FAD centre of the enzyme (Fig.8.1). In view of the presence of XOR on the outer surface of human endothelial cells (Chapter 6), the enzyme may well play an important role in signalling, particularly as the enzyme was found to be concentrated on those surfaces of cells apposed to those of neighbouring cells.

Attempts to demonstrate XOR enzymic activity on the surfaces of EA.hy 926 and L929 cells are complicated by the membrane permeability of xanthine, hypoxanthine and most reducing substrates of the enzyme.

NADH, on the other hand, is membrane impermeable and it was decided to investigate whether NADH oxidase activity of XOR was likely to participate in such signalling activities. As a preliminary exercise, experiments were designed to attempt to measure the XOR-catalysed oxidation of NADH to NAD by following the loss of absorbance at 340nm. However, this spectrophotometric measurement may not be sensitive to the very low levels of XOR present on the outer surface of the cells.

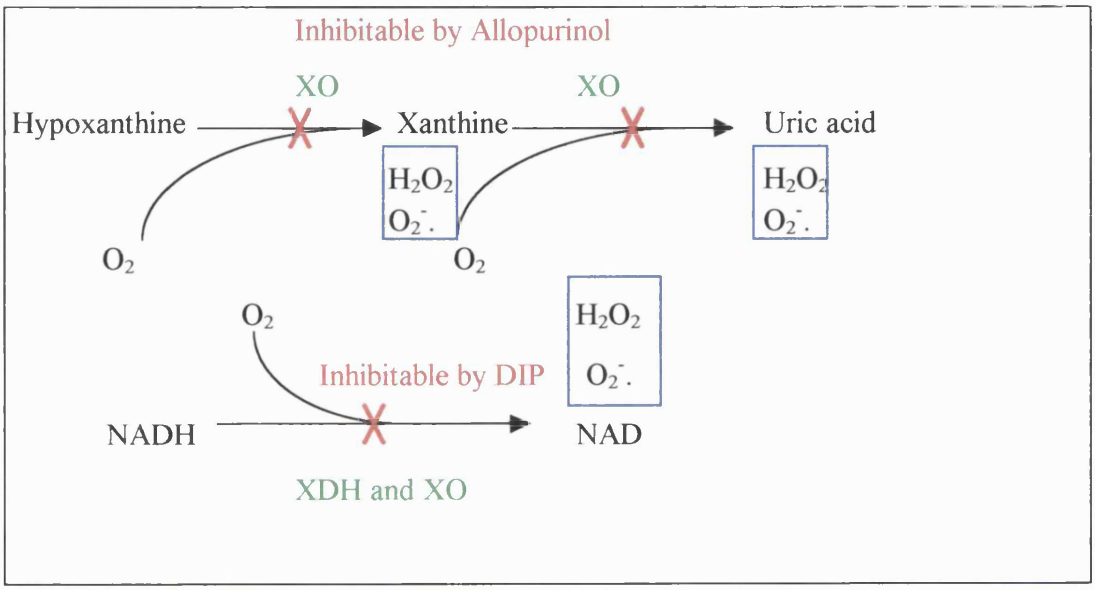


***Fig.8.1**: Schematic diagram of the inhibition by DIP and allopurinol.*
(Adapted from Zhang et al, 1998).

8.2. Methods

8.2.1. Assays

Cells were seeded at $2x10^5$ cells/ml for EA.hy 926 cells and at $1.5x10^5$ cells/ml for L929 cells. 'High' cell densities (mentioned later) correspond to double these densities. For the cells treated with allopurinol, a 1mM solution was used to produce a final concentration of 50μM in the medium (50μl). Diphenyleneiodonium chloride (DPI) was added to a final concentration of 10μM (stock solution, 1mg/ml in PBS). For inhibition studies, allopurinol and DPI were added directly to the medium of the cells a few hours after seeding and incubated for a total of 20h. NADH (stock solution 5mM) was added to all samples to a final concentration of 500μM (100μl) and

aliquots of 100µl were read at 340nm as described in Section 2.3.4 for 6h, apart for the first experiment in which a 5h assay was used. It was found that after 24h the A_{340} nm was close to zero; however, the cells did not look morphologically normal as only around 200 µl of medium was left. A significant decrease in NADH oxidase activity was observed after 6h, and so the experiment was stopped at that point.

8.2.2. Estimation of NADH oxidase activity

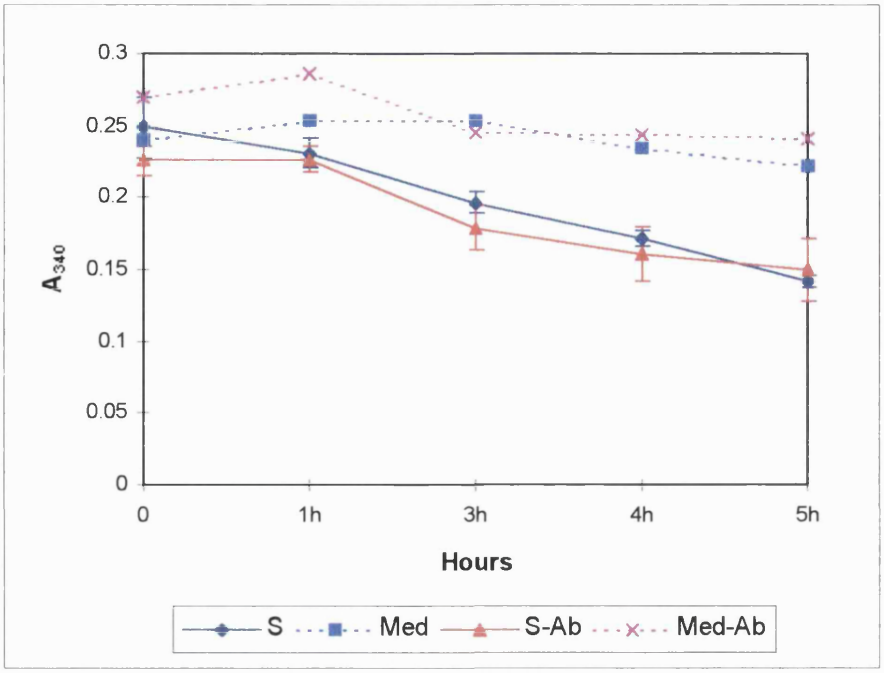
Using an extinction coefficient of $6.22 mM^{-1}cm^{-1}$ and the rate of loss of NADH observed at 340nm for each sample, the concentration of NADH oxidised was first calculated. Taking into account the dilution of the sample (100µl in 1ml, 1:10), the total volume of medium and NADH on the cell surface (1ml) and the time (6h=360min), the NADH oxidised activity was expressed in µmoles NADH oxidised x 10^{-4} per min. Finally, an estimation of the quantity of NADH found per number of cells was carried out, using the fact that V (µmol min^{-1} mg^{-1}) is equal to 0.3 for the human XDH (Sanders *et al*, 1997).

8.3. Results

8.3.1. NADH oxidase activity on the outer surfaces of EA.hy 926 cells

NADH was directly added in the medium where it is expected to remain extracellular (Eisenthal, 1998), and the time course of decrease in A_{340} was followed over 5h (Fig.8.2 A). A control experiment was carried out by adding NADH directly to cell-free medium and following the A_{340}, which did not change. Affinity purified anti-human XOR antibodies were incubated with the cells. The aim was to block XOR and check if NADH oxidation was inhibited. As shown in Fig.8.2 A (S-Ab), there was no inhibition of NADH depletion. This might be due to the fact that the incubation with the antibodies was not done in medium and the cells were starting to detach from the wells. Effectively, the antibodies have to be incubated with the cells for a few hours, and the cells were not fixed as usually done during the procedure (Section 2.7). This fact increased the chance of cells detaching from the bottom of the well. Moreover, the experimental conditions were different between the cells treated with antibodies

A)



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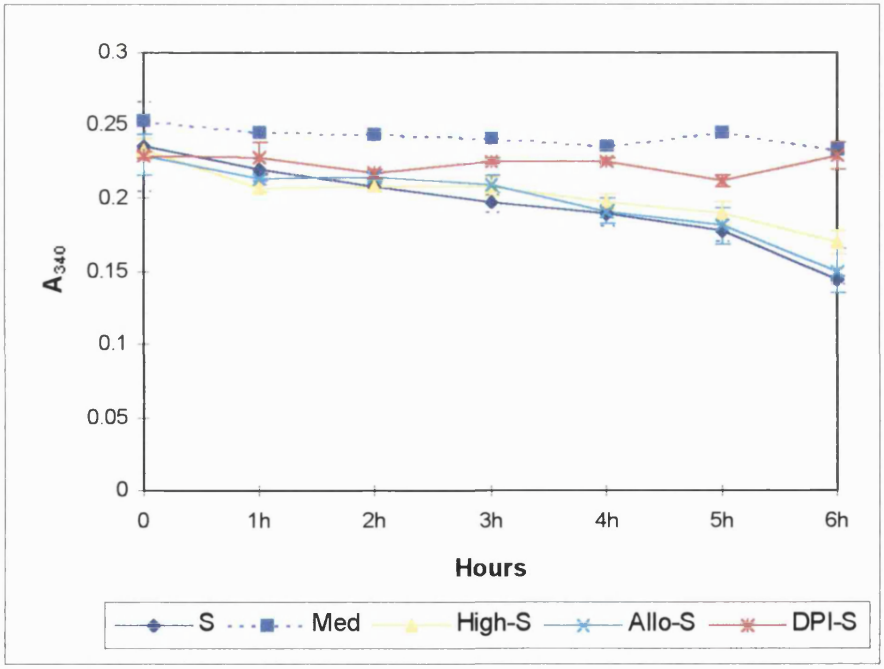


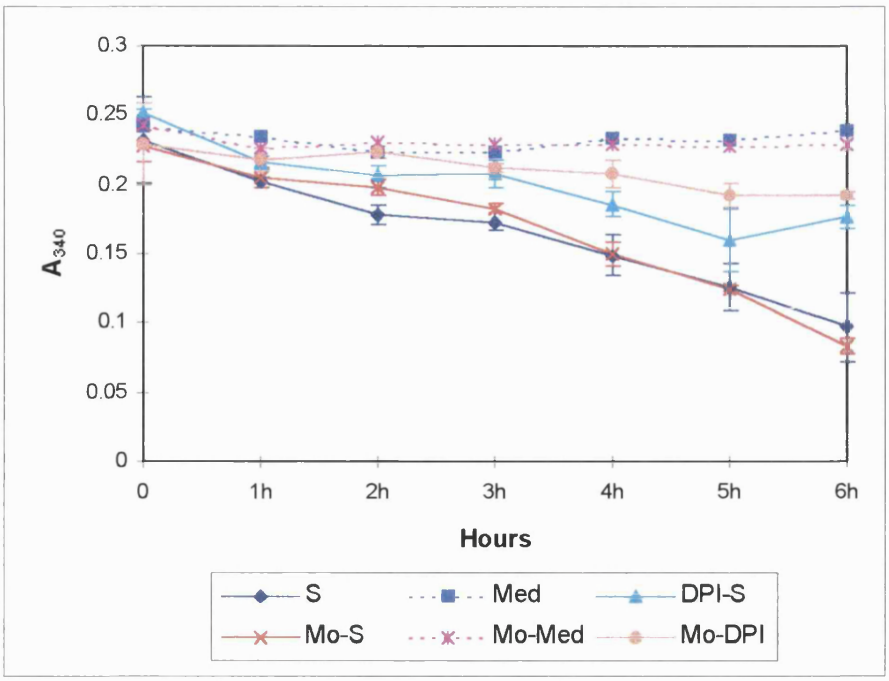
Fig.8.2: ***NADH oxidase activity on the outer surface of EA.hy 926 cells.***
The decrease of A_{340} absorbance was spectrophotometrically followed against time. S corresponds to cells alone; S-Ab, addition of affinity-purified antibodies prior to addition of NADH. High S corresponds to cells set at double density. Allo-S and DPI-S are cells which have been treated with respectively allopurinol and DPI for 20h prior to addition of NADH. Med and Med-Ab are controls containing cell-free medium and NADH. NADH was added to all samples at t=0 and aliquots of each samples were spectrophotometrically read at 340nm. Values are quoted as mean ± SEM; n=3.

and the other cells. No conclusion can be drawn from the results obtained with cells incubated with antibodies. Experiments were then carried out with cells set at higher density, in which NADH oxidase activity was expected to be proportional to the number of cells present in each well. Therefore, a faster disappearance of NADH was expected. However, this experiment was inconclusive. As previously illustrated in Fig.3.5, the cell growth pattern was disturbed when EA.hy 926 cells were set at higher density. Similar events occurred in this case, in which the cells were starting to grow on top of each other and were washed off during the first wash of the medium (Section 2.3.4). Inhibitors were incubated with the cells for 20h prior to the addition of NADH, allopurinol serving as a negative control. Allopurinol only reacts with the molybdenum centre of the enzyme and so will not affect the NADH oxidase activity. The results found (Fig.8.2 B, Allo-S) were consistent with the theory as no inhibition of the NADH oxidase activity was observed. As shown in Fig.8.2 B (DPI-S), no decrease in A_{340} absorbance was found in cells treated with DPI. DPI appeared to inhibit 90% of the NADH oxidase activity in EA.hy 926 cells (Fig.8.2 B).

8.3.2. Activity on the outer surfaces of L929 cells

Surface NADH oxidase activity was also determined on L929 cells. As described in Chapter 7, L929 cells only express xanthine oxidase activity in the presence of high levels of molybdate. It was found that the cells, whether treated or not with molybdate, induced a similar rate of NADH depletion, in terms of the rate of decrease in absorbance at 340nm. As NADH is dependent on the FAD centre rather than the Mo, this finding is to be expected, although the similar rates indicate that addition of Mo did not lead to increased expression of XOR protein. The controls consisting of cell-free medium with or without molybdate showed, as expected, no NADH depletion (Fig.8.3). DPI inhibited NADH oxidase activity in all cells. This inhibition appeared to be slightly more efficient in cells to which sodium molybdate had been added (Fig.8.3 A, DIP-S and Mo-DPI). Cells were also seeded at double the density of normal samples. It was observed that the disappearance of NADH was quicker in this case (Fig.8.3 B).

A)



B)

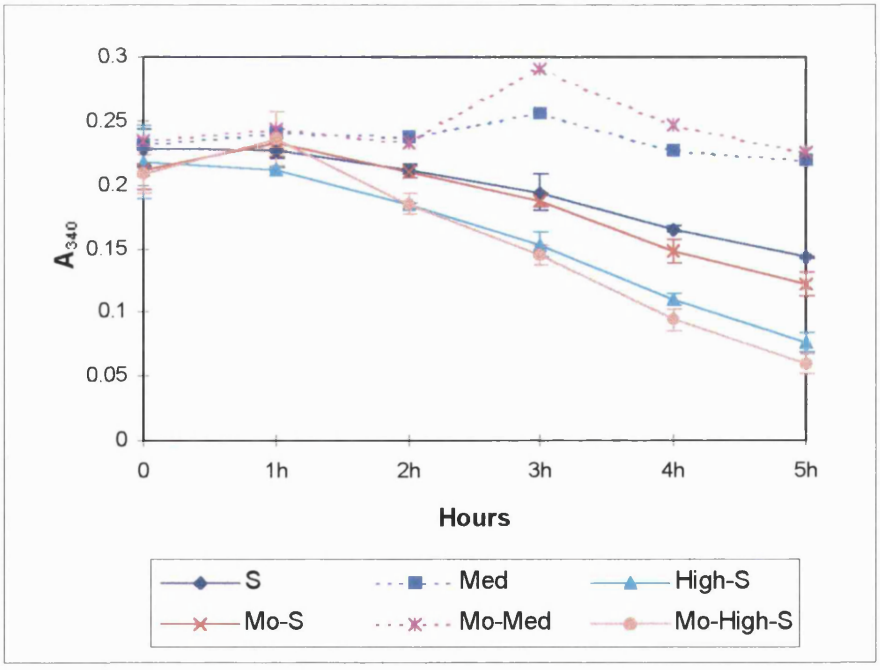


Fig.8.3: ***NADH oxidase activity on the outer surface of L929 cells.***
The decrease of A_{340} absorbance was spectrophotometrically followed against time. Mo symbolises the molybdate added to the medium of the cells. S corresponds to cells alone, High-S and Mo-High-S to cells set at double density. Mo-DPI and DPI-S are cells which have been treated with DPI for 20h prior to addition of NADH. Med and Mo-Med are controls containing cell-free medium and NADH. NADH was added to all samples at t=0 and aliquots of each samples were spectrophotometrically read at 340nm. Values are quoted as mean ± SEM; n=3.

8.3.3. Interpretation of the NADH oxidase experiments

From Table 8.1, it can be concluded that the level of NADH oxidase activity per cell is higher in L929 cells compared to EA.hy cells. These results confirm the subjective impressions from immunofluorescence experiments (Chapter 6 and 7). The addition of molybdate did not induce any change in NADH oxidation. Concerning the samples where cells were at double the density, as previously explained, the EA.hy 926 cells seeded at higher densities were starting to detach, so the result obtained is not reliable. However, the double density samples for L929 cells gave the expected results with an increase of the quantity of NADH enzyme on the surface of the cells of x1.7 fold and x1.9 fold (Table 8.1).

Samples:	A_{340} rate/hours	µmoles NADH oxidised 10^{-4}/min.	µg NADH oxidase /number cells.
EA.hy 926 cells.	0.1/5h	5.33	1.7/2x10^5 cells.
EA.hy 926 cells.	0.089/6h	3.97	1.32/2x10^5 cells.
High EA.hy 926 cells.	0.063/6h	2.82	0.94/4x10^5 cells (cells came off).
L929 cells.	0.13/6h	5.8	1.93/1.5x10^5 cells.
L929-Mo cells.	0.142/6h	6.38	2.12/1.5x10^5 cells.
L929 cells.	0.075/6h	4	1.3/1x10^5 cells.
High L929 cells.	0.1225/6h	6.56	2.19/3x10^5 cells.
L929-Mo cells.	0.075/6h	4	1.3/1.5x10^5 cells.
High L929-Mo cells.	0.13756h	7.37	2.45/3x10^5 cells.

Table 8.1: Quantity of NADH oxidase per 10^5 cells.
Using the extinction coefficient of 6.22mM^{-1}cm^{-1} and the rate of disappearance of NADH observed at 340nm, the loss of NADH was calculated per minute (Section 8.2.2). Finally, according to the kinetic parameter for NADH oxidation obtained by Sanders et al (1997), V (µmol min^{-1}mg^{-1}) for the human XDH is equal to 0.3. This value was used to calculate the quantity of NADH enzyme/number of cells (from Results in Fig.8.2 and Fig.8.3).

8.4. Conclusions

NADH oxidase activity was found on the surface of human endothelial cells and on mouse fibroblasts. This activity can be inactivated by DPI, a NADH inhibitor but not by allopurinol.

Although, it was not proven that any of the NADH activity was specific to XOR, none of the results refute this hypothesis and are consistent with it. Allopurinol interacts with the Mo centre of XOR and should not affect the FAD centre which is specific for the NADH oxidase activity. Estimates of the amount of NADH activity are, however, very high and other enzymes than XOR may well contribute to the NADH oxidase activity observed. As the cells were unpermeabilised and morphologically normal, other NADH oxidase activities must result from enzymes also present on the outer surface of the cells. Such activities have been reported, for example, NADH oxidase activity was found on the external surface of the plasma membrane of HeLa cells and other cancer cells (Morre *et al*, 1998). Various endothelial cells also express NADH and/or NADPH linked oxidases on their surfaces (Davies, 1995).

DPI inhibits the NADH activity but it is not a specific inhibitor of XOR. To prove that XOR is responsible for the NADH activity present on the outer surface of the cells, a specific inhibitor of the FAD centre of XOR would be necessary. For the moment, no such inhibitor has been found.

Although the presence of NADH oxidase activity on the outer surface of the cells neither proves that it is wholly or partly due to XOR nor that XOR is active, these experiments were an important first step in investigating a possible NADH oxidase of XOR on the surface of cells. They show that NADH oxidase activity is present and further work is required to determine to what extent XOR is responsible for it.

CHAPTER 9
GENERAL DISCUSSION

EA.hy 926 cells have been used as a model system in which to examine the role of XOR in human cells. The cells were shown to express levels of XOR enzymic activity that were low, albeit somewhat higher than those found in human epithelial cells, HB4a (Page *et al*, 1998). As mentioned in the Introduction, human XOR might be tissue-specific, with 'high activity' enzyme limited to a number of tissues (liver and intestine, for example) and 'low activity' enzyme, similar to that in breast milk, in most other tissues. This raises the question as to the function of the apparently predominant low-activity human XOR. It is conceivable that the enzyme might be subject to some forms of post-translational activation (Harrison, 1997b). Desulpho-sulpho enzyme conversion of bovine milk XOR has been effected *in vitro* by incubation of reduced enzyme with sulphide ions (Massey and Edmondson, 1970), and *in vivo* enzymic activation in response to increased protein diet has been attributed to desulpho-sulpho conversion in chickens (Itoh *et al*, 1978) and rats (Furth-Walker and Amy, 1987). Evidence of a similar post-translational up-regulation of XOR was found in HB4a cells in response to cytokines, particularly interferon-γ (Page *et al*, 1998).

XOR enzymic activity in EA.hy 926 cells followed a characteristic pattern as freshly seeded cells grew to confluence. Following a lag phase of four days, enzymic activity rose sharply (54% increase), achieving a plateau value as the cells reached confluence. Some four days later, another sharp rise (50% increase) occurred a few days before the cells began to peel away from the substrate and die. A similar rise in XOR activity after cells reached confluence was also observed, but not commented on, in BAECs (bovine aorta endothelial cells) in a study of oxygen-mediated cell injury (Panus *et al*, 1992).

An obvious question is why the specific XOR activity drops to zero when cells expressing high XOR activity are split for subculture. It could be that the specific expression in the cells is dependent on the cell cycle or cell contact, as mentioned in

Chapter 6. If *de novo* synthesis of XOR ceases at low cell density, then it is possible that turnover of existing enzyme would suffice to eliminate activity on reseeding. Alternatively, it is conceivable that activity is downregulated at a post-translational level in ways discussed above. The use of ELISA will help to clarify this and other questions relating to expression of XOR in EA.hy 926 cells during a time course.

The fact that a second rise in activity was observed as the cells are starting to die is also of particular interest. Addition of allopurinol, the best characterised inhibitor of XOR, did not result in any changes either in cell morphology or in percentage of cell viability, so gave no clue as to the relationship between XOR and growth cycle. Nevertheless, ROS have been implicated in apoptosis (Khan and Wilson, 1995), which can be induced by a wide range of factors, including radiation, growth factor removal, glucocorticoids, elevation of Ca^{2+} etc. In all these cases, mammalian cell death can be prevented by the expression of the proto-oncogene *bcl-2* (Linette and Korsmeyer, 1994). *Bcl-2* is localised in the endoplasmic reticulum, on the outer membrane of mitochondria, and on nuclear membranes, also sites of ROS generation (Korsmeyer *et al*, 1995). Possible antioxidant functions of *bcl-2* have been examined by different groups. It was found that *bcl-2* protected cells from H_2O_2-induced damage, and prevented lipid peroxidation or DNA fragmentation (Hockenbery *et al*, 1993). Suzuki and coworkers (1997) developed an experimental model for apoptosis in endothelial cells by using snake venom, which was found to generate intracellular ROS leading to apoptosis. More recently, a Japanese team showed that ROS generated by xanthine and xanthine oxidase triggered cell death associated with nuclear condensation and DNA fragmentation in cerebellar granule neurons, which were protected by catalase, but not by superoxide dismutase (Satoh *et al*, 1998). The implied role of H_2O_2 is interesting in view of the results discussed below. In our system, it is not clear if EA.hy 926 cells undergo apoptosis in culture. The use of apoptosis kits might give us an indication. Further experiments preventing the expression of XOR in the cells by addition of catalase or by changing medium should also help to understand the relationship between XOR and apoptosis. Moreover, colocalisation of *bcl-2* and XOR could be investigated using the confocal microscope.

XOR was found on the outer surface of endothelial cells, concentrated in areas where cells were apposed to or extending towards neighbouring cells. These observations might suggest a role for XOR in social interactions, particularly involving ROS. ROS are known to play a role in the modulation of mammalian cell proliferation (Burdon and Rice-Evans, 1989; Burdon 1992; Burdon, 1995), and free radical-derived species can have a significant modulatory influence on growth in a variety of cell types (hamster and mouse fibroblasts, human primary fibroblasts and mouse epidermal cells) when added exogenously to the culture medium (Burdon and Gill, 1993).

Evidence for a secreted signal was found when medium was changed four times a day for 48h. Although the cell morphology was not altered, the XOR activity was significantly decreased by nearly 90%. This result strongly suggests that XOR expression is dependent on agents secreted by the cells in the medium. However, attempts to induce XOR expression in endothelial cells by addition of conditioned medium were unsuccessful. In attempts to further investigate possible signals, extracellular agents (SOD, catalase and H_2O_2) were added to EA.hy 926 cells and their influence on XOR expression was checked. Catalase induced a significant decrease of XOR activity, but direct addition of H_2O_2 to EA.hy 926 cells affected neither cell viability nor the specific expression of XOR in the cells. These results are similar to the ones found by Phan *et al* (1989), who showed that XO activity was not increased by H_2O_2 in rat pulmonary artery endothelial cells. It is possible that the concentration of H_2O_2 used was not at the right level to induce any effect on XOR activity. Addition of exogenous SOD to the cells also had no effect on the specific XOR activity.

Low levels of ROS may be involved not only in intercellular signalling but also in intracellular redox signalling events, for example controlling gene expression. From the confocal microscope studies, XOR was shown to be distributed not only on the outer surface of endothelial cells but also in the cytoplasm, where it is concentrated in the perinuclear region. The outer membrane of the nuclear envelope is continuous with the membranes of the endoplasmic reticulum (Loewy *et al*, 1991). ROS are increasingly thought to be important as agents of signal transduction and in particular in the activation of nuclear transcription factors, such as NF-*k*B.

The localisation of XOR on or near the nuclear membrane would be relevant to such a role. From these preliminary results, a sketch can be drawn concerning the activation of XOR and the possible role of XOR in cell signalling (Fig.9.1).

The presence of extracellular XOR raises the question of how the enzyme is secreted from the surface of the cells. XOR was found to have no signal peptide and not to be glycosylated (Ichida *et al*, 1993 and Xu *et al*, 1994). It is therefore quite likely that XOR is not secreted by the classical pathway. In 1993, Kuchler reviewed proteins that lack a typical hydrophobic N terminal leader sequence and which are secreted. Among these different proteins are cited interleukin-I and factor XIIIa. XOR may not be involved in the endoplasmic reticulum-Golgi route but instead in the ABC resident endosomal transporter which is thought to be involved in the alternative pathway (Kuchler, 1993). This could also explain the presence of the enzyme concentrated around the perinuclear region of the cells.

Further evidence of the presence of XOR on the surface of the cells was provided by the demonstration of NADH oxidase activity. This activity is inhibited by DPI, a specific inhibitor of all flavoenzymes, and not all of this NADH oxidase activity can be attributed to XOR. An increasing number of NAD(P)H oxidases are being detected on mammalian cell surfaces. Such oxidase complexes appear to have a role similar to that of phagocytic cell NADPH oxidase, and may generate a constant stream of O_2^- to maintain a local environment that discourages bacterial/fungal infection (Davies 1995). An NADPH oxidase that releases extracellular superoxide was found in the rabbit aorta (Pagano *et al*, 1993; 1995). Similar NADH oxidases were found to be the predominant source of superoxide in bovine endothelial and vascular smooth muscle cells (Mohazzab *et al*, 1994; Mohazzab and Wollin, 1994), while the major source of vascular superoxide ion and hydrogen peroxide was reported to be a membrane bound NADH oxidase (Harrison, 1997a). In many cases, it is not clear to what extent reported NADH oxidase activities can be attributed to XOR. This possibility is often supposedly eliminated by failure of allopurinol to block that observed activity. In fact, XOR-catalysed NADH oxidase activity is not inhibited by allopurinol or by most other classical XOR inhibitors which act at the molybdenum site (Sanders *et al*, 1997).

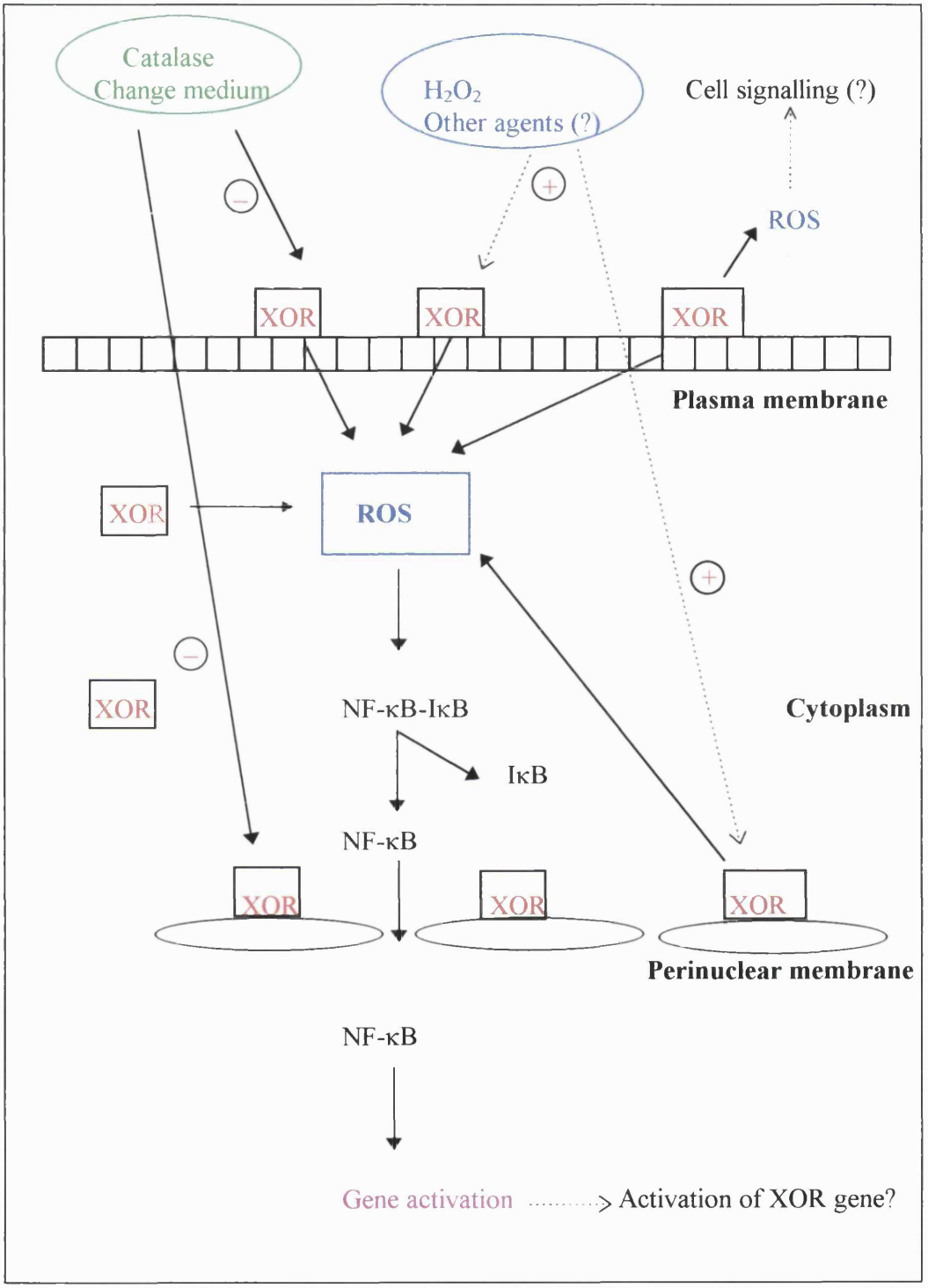


Fig.9.1: ***Potential role of XOR and ROS in cell signalling based on the results discussed in this thesis (other factors can cause the dissociation of NF-κB or the production of ROS, but for simplicity these were not shown here).***

Bayraktutan and coworkers (1998) have recently suggested a role for NAD(P)H oxidases in cellular signalling pathways in addition to mediating the cellular injury associated with excessive free radical generation.

The discovery of XOR on the surface of endothelial cells and other cells could help us to understand the role of XOR in inflammation. As mentioned in the General Introduction, a proposed endothelial cell trigger mechanism was presented by Bulkley's team (Ratych *et al*, 1987; Bulkley, 1997). This model is based on the fact that the endothelial/cell-based XOR system and the neutrophil-based NADPH oxidase system each generates reactive oxidants from a different source, and these two mechanisms combine to mediate reperfusion injury. The initial production of reactive oxidants by endothelial XOR triggers the arrest-trapping, activation and subsequent diapedesis of circulating neutrophils. While activation of lymphocytes and rolling have been well studied, little is known of the diapedesis process (Springer, 1990; Baumann and Gauldie, 1994). XOR situated at the junction of two endothelial cells could attract the activated lymphocytes. Obviously, this model depends on the presence of cell surface XOR, which was indeed briefly reported in abstracts from the same team (Bulkley, 1991; Schiller *et al*, 1992). Following the publication of our results on the distribution of XOR in endothelial and epithelial human cells (Rouquette *et al*, 1998), Vickers and coworkers (1998) published their data providing existence for the presence of XOR on the outside surface of primary cultures of bovine (BAECs) and porcine (PAECs) aortic endothelial cells. This finding leads one to think that the localisation of XOR on the outer surface of cells is not specific to human endothelial and epithelial cells.

The occurrence of endothelium-derived XOR might explain systemic complications such as acute respiratory distress syndrome (ARDS). ARDS is associated with activation of neutrophils and therefore with release of free radicals and ROS in the pulmonary circulation (Chabot *et al*, 1998). Quinlan and coworkers (1997) showed that patients with ARDS had higher levels of xanthine and hypoxanthine compared to controls. Another team reported that xanthine oxidase activity was associated with arterial blood pressure in spontaneously hypertensive rats.

Xanthine oxidase activity was inhibited by a tungsten diet and by administration of (-) BOF 4272. The inhibitory effects of the tungsten diet on the increasing blood pressure and arteriolar tone in spontaneously hypertensive rats was reproducible by administration of (-) BOF 4272 (Suzuki *et al*, 1998). It is interesting that NF-κB is activated in lungs of patients with ARDS. In this model, blood loss was partly through xanthine oxidase-dependent mechanisms (Moine *et al*, 1997). These facts suggest that XOR and ROS are involved in important diseases and syndromes.

Concerning antibodies to XOR in blood, Benboubetra and coworkers (1997) found that levels of IgM anti-XOR autoantibodies are remarkably high in normal human serum. High levels of anti-XOR antibodies can be seen as advantageous, as they could neutralise and/or clear endogenous XOR from the circulation (Benboubetra *et al*, 1997). The presence of circulating anti-XOR autoantibodies is also relevant in considerations of XOR on the surface of vascular endothelial cells. The enzyme has been shown to bind reversibly and with high affinity to glycosaminoglycans on the surface of endothelial cells (Radi *et al*, 1997). ROS generated by glycosaminoglycan-bound XOR might be expected to diffuse into the intravascular compartment and react with circulating blood elements. White and coworkers (1996) showed that levels of XOR are increased in the plasma of cholesterol-fed rabbits and that this circulating XOR binds to heparin-binding sites on the vessel wall, where it produces excess superoxide. Inhibition of XOR with oxypurinol improves endothelium-dependent vascular relaxation (White *et al*, 1996).

A recent finding from our laboratory is relevant to the possible role of endothelial XOR. The enzyme was shown to catalyse the reduction of organic and inorganic nitrate to nitric oxide (NO) under hypoxic conditions and in the presence of NADH, suggesting a vasodilatory role in ischaemia (Millar *et al*, 1998). These findings could also be relevant to the inflammatory response, in which the potential for simultaneous release of NO and superoxide suggests a role for XOR-generated peroxynitrite as a destruction agent in diseases involving hypoxic episodes.

The results obtained with L929 mouse fibroblastic cells are worthy of brief comment. These cells have been shown to contain inactive XOR protein which can be activated by addition of molybdenum salts to the culture medium (Falciani *et al*, 1994). In the present studies, the enzyme was shown to be localised on the outer membrane of the cells and in the cytoplasm concentrated around the perinuclear region, as found for EA.hy 926 cells. Moreover, NADH oxidase activity was also detected on the surface of L929 cells which could in part be attributed to XOR. In the previously reported work, although XOR-specific mRNA was detected in L929 cells prior to molybdenum addition, the inactive protein was not actually determined. We now know that the inactive protein is not only present in the cells but is distributed in a manner entirely analogous to that in EA.hy 926 cells.

Most of this thesis has been directed to an investigation of the occurrence and potential roles of XOR in human endothelial cells. The enzyme is clearly present in EA.hy 926 cell line and appears to exist in a form with low specific activity, like that in human milk. The relationship between expression of XOR activity and the growth of endothelial cells is intriguing, and particularly the sharp rise in activity shortly before death of the cells merits further investigation. The most important finding is the localisation of XOR on the outer surface of the cells with important implications for the role of the enzyme in the inflammatory response. The question as to how the enzyme is secreted from the cell remains open. Study of NADH oxidase on the surface of the endothelial cells indicated the presence of activity that could not all be attributed to XOR, and again needs further investigation.

Overall, EA.hy 926 cells have been shown to be a useful model for study of the role of XOR in human endothelial cells and a number of potentially interesting approaches for further work have been suggested.

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APPENDIX

Appendix I: XOR in endothelial cells

Cells/tissues studied.	XOR activity.	Method of detection.	References.
Bovine EC and tissues.	Positive in capillary EC from liver, heart, lung and intestine.	Immunolocalisation.	Jarasch *et al*, 1981.
Selection of bovine cell cultures and tissues.	High amount: mammary gland epithelial and capillary EC, and liver.	Enzymic radioassays, measuring uric acid.	Bruder *et al*, 1983.
Capillary endothelial cells and tissues from bovine and human.	Positive: capillary EC of many tissues (liver, heart, lung, kidney). But not in other epithelial, EC, and mesenchymal cell types.	Histochemistry and radioimmunoassay.	Jarasch *et al*, 1986.
Rat pulmonary artery endothelial cells (RPAEC).	Increase in XO activity after I/R but decrease in XDH activity. XOR is stable.	Spectrophotometric assay measuring amount of uric acid.	Ratych *et al*, 1987.
Bovine pulmonary arterial endothelial cells.	High XO activity in EC.	HPLC: uric acid peak detected at 290nm.	Rodell *et al*, 1987.
Rat pulmonary artery endothelial cells (RPAEC).	High activity found.	HPLC reverse phase. Quantification of radioactive uric acid.	Phan *et al*, 1989.
Normal rat heart.	XO activity in EC of capillaries, small blood vessels and interstitial cells.	Histochemistry and spectrophotometric assay of urate (295nm).	Samra *et al*, 1991.
Bovine brain EC.	Bovine brain EC had increased level of XO & XDH/ whole brain homogenate.	HPLC: formation of uric acid.	Terada *et al*, 1991.

Pulmonary artery endothelial cells (PAECs).	No XDH or XO activity in PAEC exposed to normoxia or hypoxia for 5, 26, or 50h.	Spectrofluorometric assay.	Bhat *et al*, 1992.
Rat pulmonary endothelial cells: rat lung microvascular cells (LMVC) and pulmonary artery endothelial cells (PAEC).	Control low activity. IFN-γ: inducer of XDH/XO activity in LMVC & PAEC. Upregulation at transcriptional level.	Fluorimetric assay.	Dupont *et al*, 1992.
Unfixed cryostat sections of various rat tissues.	Activity in sinusoidal cells of liver, EC in various tissues.	Histochemical method.	Kooij *et al*, 1992.
HUVECs.	XO activity found after hypoxia. Undetectable basal state.	Fluorimetric assay.	Michiels *et al*, 1992.
HUVECs.	H/R: decrease XDH and increase XO.	Measuring uric acid at 293nm.	Palluy *et al*, 1992.
Bovine aortic endothelial cells (BAEC).	XO/XDH found with a maximum on day 9.	Pterin assay on fluorimeter.	Panus *et al*, 1992.
Bovine pulmonary microvascular endothelial cells (BPMVE).	Hypoxia 24h: double XO+XDH activity/normoxia. Hypoxia 60min. no effect/normoxia.	HPLC: C18 reverse phase column→ peaks of uric acid.	Partridge *et al*, 1992.
Cultured bovine pulmonary artery endothelial cells.	Exposure to decreasing O_2 tension: increase XDH/XO activity.	HPLC. Quantification of uric acid.	Terada *et al*, 1992.
Human and rat tissues.	High activity: EC & Kupffer cells from rat and human liver. Low activity: liver parenchymal cells in rat.	Quantitative histochemical.	Frederiks *et al*, 1993a.

Rat tissues: liver and small intestine.	Rat liver: very low XO activity, with higher activity in EC. High activity in epithelial layer of small intestine. 60-120min *in vitro* ischaemia, no effect on activity in liver.	Histochemical method. In vitro ischaemia.	Frederiks *et al*, 1993b.
Human tissues.	Staining: smooth muscle cells of larger vessels in human cardiac and skeletal muscle. XO in human aorta, pulmonary artery, umbilical cord. Weak positive in EC of capillary, small venules and arterioles in human cardiac and skeletal but negative in EC lining inside larger vessels.	Immunohisto--chemistry.	Hellsten-Westing, 1993
Human liver and tissues.	Localisation: liver cells, duodenal mucosa & endothelial lining cells from heart, kidney, brain, aorta, lung, mesentery, hepatic tissue. No protein found in tissue cytosol, kidney, heart, lung and brain.	Immunohisto--chemical localisation by polyclonal Ab αXO.	Moriwaki *et al*, 1993.
HUVECs.	Control-XO+XDH activity found. Add IFN-γ (1000U/ml; 24h) no change.	Uric acid formation by HPLC.	Terada *et al*, 1993.

-Porcine pulmonary artery EC (PAEC) -HUVECs -bovine pulmonary artery smooth muscle cells (PASMC) -bovine PAEC -Rat epididymal fat pad EC -Rat lung.	No activity in porcine PAEC and HUVECs even after exposure to hypoxia. The other cells show XO and XDH activity.	Fluorimetric assay.	Hassoun *et al*, 1994.
-Bovine aorta EC (BAEC) -Human aorta EC -Human coronary artery EC -Human microvascular EC -Porcine aorta EC -Rat aorta EC -Rat pulmonary artery EC.	No activity found except in BAEC and pulmonary artery EC.	XOR activity: fluorimetrically by oxidation of pterin to isoxanthopterin.	Paler-Martinez *et al*, 1994.
Hepatocytes EC, Kupffer cells and hepatocytes from rat liver.	XDH+XO activity in EC, Kupffer cells and hepatocytes. Hypoxia: conversion XDH to XO in the three cell types.	Spectrophotometric assay of urate (295nm).	Wiezorek *et al*, 1994.
Human aortic endothelial cells (HAECs).	XO and XDH were measurable.	Spectrophotometer. 295nm, uric acid production in presence of xanthine.	Zweier *et al*, 1994.
Porcine artery endothelial cells (PAEC).	No detectable XDH/XO. Hypoxia: reduction intracellular and release H_2O_2. No increase ROS production during reperfusion.	H_2O_2: spectrofluorometric assay.	Yang *et al*, 1995.
Cultured endothelial cells isolated from bovine arteries.	EC expressed XDH and XO activity. Effect of activated neutrophils on the conversion of XDH to XO.	HPLC. Quantification of uric acid at 290nm.	Wakabayashi *et al*, 1995.

Cultured bovine aortic endothelial cells (BAEC).	Doubling XDH/XO activity exposed to hypoxia and H/R.	Fluorimetric assay.	Poss *et al*, 1996.
Rat tissues.	High activity EC liver, kidney, hepatocytes of liver, myocytes of heart, smooth and striated cells of muscle, acinar cells of pancreas, epithelial cells of ducts and small intestine and colon.	Localisation of SOD. Light microscope and Immunohistochemis -try.	Frederiks *et al*, 1997.
Human microvascular endothelial cells.	Increase of XO in human muscle associated with 2nd inflammmatory processes.	XO activity: chemiluminescence. Distribution: immunohisto-chemistry.	Hellsten *et al*, 1997.
Bovine pulmonary artery endothelial cells.	Post I/R: intracellular ROS formation increased to 148%. EC exposed to I/R: allopurinol and nitric oxide synthase reduced intracellular ROS formation.	Fluorimetric assay to measure ROS.	Zulueta *et al*, 1997.

***Legend**: EC: endothelial cells; H/R: hypoxia/reoxygenation; I/R: ischaemia reperfusion; ROS: reactive oxygen species.*

Appendix II: publication

Rouquette, M, Page, S., Bryant, R., Benboubetra, M., Stevens, C.R., Blake, D.R., Whish, W.D., Harrison, R., Tosh, D. (1998) *FEBS letters* **426**, 397-401. "Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture."

FEBS 20152 FEBS Letters 426 (1998) 397–401

Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture

Magali Rouquette[a], Susanna Page[a], Richard Bryant[a], Mustapha Benboubetra[a], Cliff R. Stevens[b], David R. Blake[b], William D. Whish[a], Roger Harrison[a,*], David Tosh[a]

[a] *Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK*
[b] *Department of Postgraduate Medicine, University of Bath, Bath BA2 7AY, UK*

Received 19 March 1998

Abstract Subcellular localisation of xanthine oxidoreductase (XOR) was determined by indirect immunofluorescence using confocal microscopy in human endothelial and epithelial cell lines and in primary cultures of human umbilical vein endothelial cells. XOR was diffusely distributed throughout the cytoplasm but with higher intensity in the perinuclear region. In non-permeabilised cells, XOR was clearly seen to be asymmetrically located on the outer surfaces, showing, in many cases, a higher intensity on those faces apposed by closely neighbouring cells. Such specific distribution suggests a functional role for the enzyme in cell-cell interactions, possibly involving signalling via reactive oxygen species

Key words: Xanthine oxidoreductase; Immunolocalization; Endothelial; Epithelial; Human

1. Introduction

Xanthine oxidoreductase (XOR) is a molybdenum-containing flavoenzyme that catalyses the hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid in the latter stages of purine catabolism [1]. In mammals, it occurs in two interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204) and xanthine oxidase (EC 1.1.3.22). Both forms of the enzyme can reduce molecular oxygen, although only the dehydrogenase form can reduce NAD, which it prefers as an electron acceptor. Reduction of oxygen leads to superoxide anion and hydrogen peroxide and it is the potential to generate these reactive oxygen species that has led to widespread interest in the enzyme as a pathogenic agent in many forms of ischaemia-reperfusion injury [2]. More recently, reactive oxygen species are being increasingly cited as intermediates in normal signal transduction pathways [3,4].

XOR is widely distributed, being particularly rich in mammary epithelial cells and in capillary endothelium in a range of tissues [5,6]. While the enzyme is generally understood to be cytosolic, there have been very few published investigations of its precise subcellular localisation. Jarasch et al. [5] used both light and electron microscopic immunohistochemical procedures to show that XOR is located throughout the cytoplasm of bovine capillary endothelial cells. This was also found to be the case in rat liver endothelial cells [7,8]. In contrast, using immunoelectron microscopy, Ichikawa et al. [9] concluded that the enzyme was exclusively cytosolic with no significant association with intracellular organelles, including endoplasmic reticulum, Golgi apparatus, lysosomes or peroxisomes.

The apparent confusion over the subcellular location of XOR prompted us to investigate the situation in human cells. The human enzyme is of especial interest, particularly in view of questions regarding its anomalous characteristics and physiological role [10]. We have, accordingly, made use of confocal microscopy in immunolocalisation of the enzyme in human endothelial and epithelial cells in culture. We show here that XOR is present not only in the cytoplasm but also on the outer surface of all three cell types studied. Moreover, the extracellular enzyme shows a strongly polarised distribution, being in many cases concentrated on those surfaces closely apposed by neighbouring cells.

2. Materials and methods

2.1. Materials

RPMI 1640 culture medium was obtained from ICN, Costa Mesa, CA, USA. Penicillin, streptomycin and foetal calf serum (FCS) were from Life Technologies, Paisley, UK. Rabbit anti-TGN 46 antibody was a kind gift from Dr. George Banting (Department of Biochemistry, University of Bristol, UK). Rabbit anti-(bovine milk XOR) was from Chemicon International, Harrow, UK. All other reagents, unless otherwise stated, were from Sigma, Poole, UK.

2.2. Cell culture

EA-hy-926, a permanent endothelial cell line [11], was a gift from Dr. Andrew George, Imperial College School of Medicine, Hammersmith Hospital, London. The cells were routinely grown in an atmosphere of 5% CO_2/95% air in 75-cm^2 flasks at 37°C, as previously described [12]. Growth medium, RPMI 1640, containing 10% (v/v) FCS and penicillin/streptomycin [12], was changed every 3–4 days. The cells grew to form a confluent monolayer after approximately 7 days, exhibiting typical endothelial cell characteristics and were shown, by immunofluorescence (results not shown), to be positive for factor VIII, as reported by Edgell et al. [11].

HB4a is a human mammary epithelial cell line, conditionally immortalised by transfection with SV40 virus [13] and kindly donated to us by Dr T. Kamalati and Professor B. Gusterson of the Institute for Cancer Research, Royal Cancer Hospital, Sutton, UK. HB4a cells were routinely grown at 37°C in 75-cm^2 culture flasks, in an atmosphere of 5% CO_2/95% air as previously described [14]. Growth medium, RPMI 1640 containing 10% (v/v) FCS and other supplements [14], was changed every 3–4 days. Cells grew to confluence, forming a strict monolayer after 9 days, showing a characteristic 'crazy paving' appearance and stained strongly positive (results not shown) for the epithelial cell marker, cytokeratin [14].

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical veins (kindly donated by the nursing staff of the Princess Anne Wing, Royal United Hospitals, Bath) and cultured essentially as described by Jaffe et al. [15].

*Corresponding author. Fax: (44) (1225) 826779.
E-mail: bssrh@bath.ac.uk

Abbreviations: XOR, xanthine oxidoreductase; FCS, foetal calf serum; HUVEC, human umbilical vein endothelial cell; DIC, differential interference contrast

PII S0014-5793(98)00385-8

2.3. Immunolabelling of cells and confocal microscopy

Cells were seeded (approx. 2×10^5 cells/ml) in four-chambered glass slides (Nunc Inc., Naperville, IL, USA), incubated for 24 h at 37°C and washed twice with pre-warmed PBS before fixing for 20 min at room temperature with 4% (w/v) formaldehyde in PBS.

Cells were permeabilised by incubation with 0.1% (w/v) saponin in PBS for 45 min, then incubated with rabbit polyclonal anti-(human XOR) antibodies in PBS (0.02 mg/ml), containing 0.1% (w/v) saponin, 3% (v/v) normal goat serum and 1% (w/v) BSA, for 2 h at room temperature. The cells were washed three times with 0.1% (w/v) saponin in PBS before incubation, for 2 h at room temperature, with secondary antibody [FITC-conjugated goat anti-rabbit IgG (0.025 mg/ml, Jackson Immunoresearch Labs. Inc., West Grove, PA, USA)], diluted 1:100 in the same diluant as for the primary antibodies. The cells were then washed three times with PBS containing 0.1% (w/v) saponin, before removing the chambers from the slides prior to confocal microscopy.

Unpermeabilised cells were obtained and treated as above, except that saponin was omitted throughout.

The permeabilised or unpermeabilised nature of the cells was confirmed by immunolabelling with rabbit anti-TGN 46 antibody, which is specific for the trans-Golgi network (results not shown) [16].

Images were collected on a confocal laser-scanning microscope (LSM 510, with either ×40 1.30 NA or ×63 1.40 NA apochromatic objective; Carl Zeiss, Welwyn Garden, UK). The 488 lines of an argon laser were used for excitation of FITC.

2.4. Assay for XOR enzymic activity

Cell extracts were prepared and assayed for total (oxidase plus dehydrogenase) activity as previously described [14], using a sensitive fluorimetric procedure [17]. EA-hy-926 and HB4a cells contained 1–2 pmol isoxanthopterin/min/mg. Activity of HUVECs was below the lower limit of sensitivity of the assay (0.1 pmol isoxanthopterin/min/mg).

2.5. Heparin-Sepharose treatment of growth medium

A column (3.5 cm×1.5 cm) of heparin-Sepharose (Sigma) was washed with appropriate growth medium (30 ml) lacking FCS. Growth medium (100 ml) containing FCS (10%) was then passed through the column and collected in a sterile container. The column was washed with 25 mM sodium phosphate buffer, pH 7.4, until A_{280} reached a baseline level, and then with the same buffer containing 1 M NaCl. Protein-containing fraction (A_{280}) was assayed for XOR enzymic activity as described above.

2.6. Generation and affinity purification of rabbit polyclonal anti-(human XOR) antibodies

Antibodies were generated and affinity-purified as previously described [14]. Their specificity has been previously established by immunoprecipitation of XOR from HB4a cell extracts [14]. Incubation of HB4a cell extracts with the gel-bound specific antibodies removed 100% of XOR enzymic activity, while SDS-PAGE of the immunoprecipitate showed only the characteristic band of XOR, apart from bands attributable to the antibodies themselves [14].

3. Results

Three human cell types, including endothelial (EA-hy-926) and epithelial (HB4a) cell lines and primary endothelial (HUVEC) cells in culture, were studied using affinity-purified rabbit anti-(human XOR) antibodies (see Section 2). In all cases, immunolocalisation of XOR in permeabilised cells showed the enzyme to be diffusely distributed throughout the cytoplasm, although fluorescence in the perinuclear region was more intense (Fig. 1). Immunolocalisation of XOR in unpermeabilised cells clearly showed the presence of the enzyme on the outer cell surface, the distribution being localised to specific areas of the cell (Fig. 2). In several cases, XOR appeared to be concentrated on those parts of the surface that apposed or were extending towards neighbouring cells (Fig. 2C, arrows).

Use of commercially supplied rabbit anti-(bovine milk XOR) antibody gave the same results as those described above (results not shown).

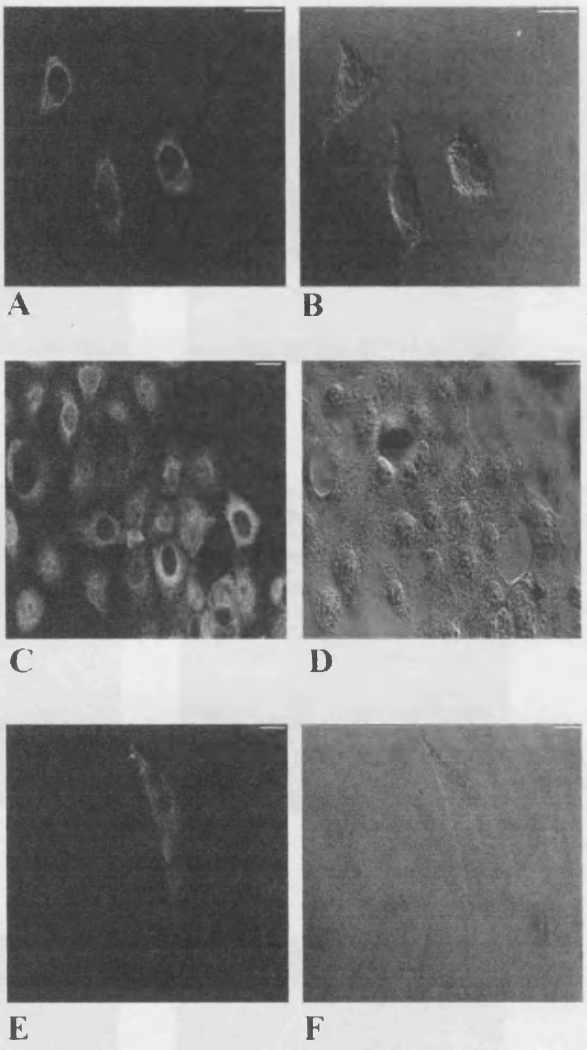

Fig. 1. Distribution of XOR in permeabilised EA-hy-926 cells (A, B), HB4a cells (C, D) and HUVECs (E, F). For experimental details see Section 2. Immunofluorescent (A, C, E) and differential interference contrast (DIC) (B, D, F) images are shown. Magnification ×630 (A, B), ×400 (C–F); bar, 20 μm.

Because of the possibility that XOR on the surface of our cells in culture originated in the growth medium (which contains FCS) the latter was assayed for XOR. Enzymic activity could not be detected by the sensitive fluorimetric procedure (see Section 2). In view of the high affinity of human XOR for heparin [18,19], we sought to concentrate any small amounts of XOR in the growth medium by passage down a column of heparin-Sepharose (see Section 2). In none of six batches of serum was XOR activity detectable by fluorescence assay when the heparin column was subsequently eluted with 1 M NaCl, conditions known to elute the human enzyme [19]. Two further control experiments addressed this issue. In the first of these, parallel cultures of EA-hy-926 or HB4a cells were grown in 75-cm^2 flasks in the corresponding growth medium that had, or had not been passed down a heparin-Sepharose column (see Section 2). In each case, cells were then seeded onto duplicate wells of glass slides as usual and subjected to

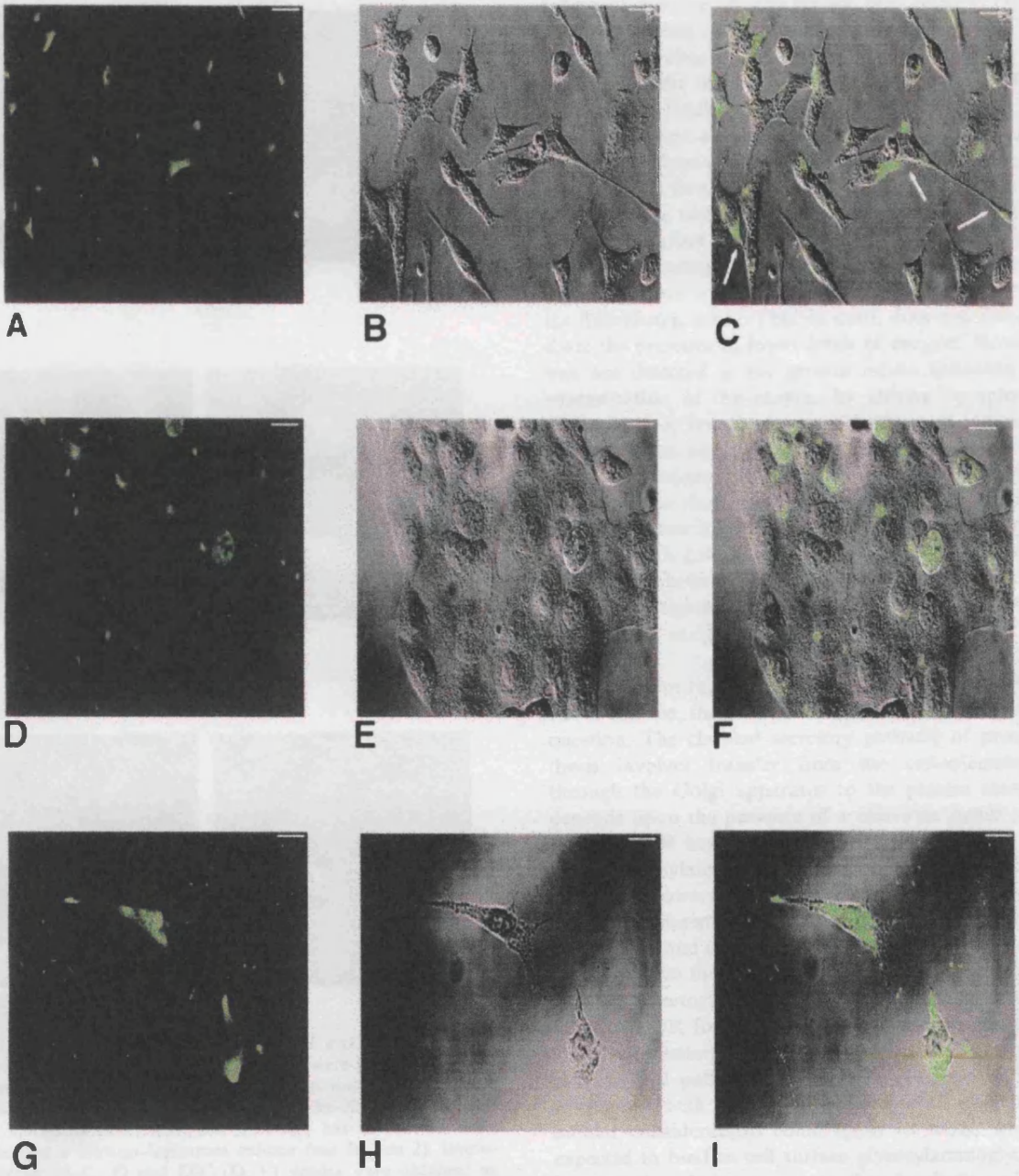

Fig. 2. Distribution of XOR in unpermeabilised EA-hy-926 cells (A–C), HB4a cells (D–F) and HUVECs (G–I). For experimental details see Section 2. Immunofluorescent (A, D, G) and DIC (B, E, H) images are overlaid (C, F, I) to emphasise the polarised distribution. Arrows (C) show examples where fluorescence is concentrated on surfaces that appose those of neighbouring cells. Magnification ×400; bar, 20 μm.

immunolabelling. No difference was apparent in the distribution or intensity of the fluorescence patterns between unpermeabilised cells grown in heparin-treated and untreated medium, nor was there any significant difference in total XOR activity in the cells, as assayed fluorimetrically. Results for EA-hy-926 cells are shown in Fig. 3C–F, in which the polarised distribution of the enzyme is again clearly seen. In the second control experiment, heparin-Sepharose beads (300 μl) were washed twice with PBS before incubation overnight, with continuous gentle agitation, either with growth medium (containing FCS), PBS or with PBS containing bovine XOR (10 μg/ml). Subsequent labelling with anti-XOR antibody showed clear immunofluorescence on the surface of the beads in the latter but not the former case (Fig. 3). Similar incubation of heparin beads with normal goat serum, used as a blocking agent in immunolabelling, also failed to lead to immunofluorescence on the surface of the beads.

4. Discussion

While reactive oxygen species are increasingly being considered as agents of signal transduction [3,4], their source is seldom clear, and XOR, with its capacity for generation of superoxide anion and hydrogen peroxide, is in many cases an attractive candidate [10]. The subcellular localisation of the enzyme is clearly relevant to its function and it is with

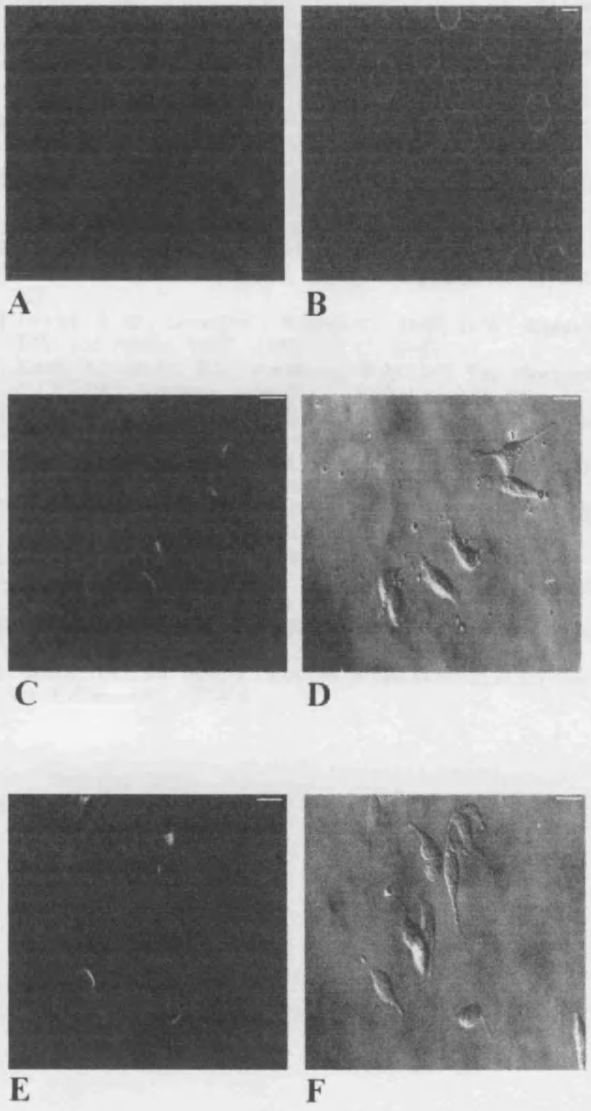

Fig. 3. Control experiments showing that cell surface XOR is not derived from growth medium. Heparin beads were incubated for 24 h with growth medium (A) or with PBS containing bovine XOR (B) (see Section 3). C, D and E, F show EA-hy-926 cells grown in medium (containing FCS), that has (E, F) or has not (C, D) been preabsorbed on a heparin-Sepharose column (see Section 2). Immunofluorescence (A–C, E) and DIC (D, F) images were obtained as described in Section 2. Magnification ×100; bar, 50 μm (A, B); ×400; bar, 20 μm (C–F).

this in mind that we examined the former in cultured human cells.

XOR is generally assumed to be a cytoplasmic enzyme, although its precise localisation is unclear, having been described as being both peroxisomal [7,8] and exclusively cytosolic [9]. In the permeabilised cells of the present study, XOR was seen to be generally distributed throughout the cytoplasm but with more intense staining in the perinuclear region. This latter localisation has not, to our knowledge, been suggested previously and has interesting implications concerning possible functions of the cytoplasmic enzyme. A perinuclear location would, for example, accord with a role for XOR as a source of reactive oxygen species that activate nuclear transcription factors, such as NF-κB [20].

XOR was clearly detected on the outer surface of unpermeabilised cells of all three human cell types studied. While extracellular localisation of XOR has previously been proposed in bovine aortic endothelial cells [21,22], our presently reported findings constitute the first detailed evidence of such a localisation in any cell type. In view of the potential importance of these results, it was necessary to eliminate the possibility that surface enzyme is derived from exogenous sources, such as, for example FCS in the growth media. Growth media did not contain levels of XOR above the limit of sensitivity of the fluorimetric assay. This, in itself, does not necessarily preclude the presence of lower levels of enzyme. However, XOR was not detected in the growth media following attempted concentration of the enzyme by chromatography on heparin-Sepharose, nor were any differences in immunolabelling detected when any of the three cell types was grown in preabsorbed medium. Moreover, growth medium for all three cells failed to show fluorescence labelling of heparin-Sepharose beads when incubated with the latter. Similar results were obtained with goat serum, routinely used as a blocking agent in immunolabelling. Finally, it is highly unlikely that cell surface XOR originated in lysed neighbouring cells, which are at low density early in their growth cycle and are essentially 100% viable.

If we accept that the extracellular XOR is indeed an endogenous enzyme, then the mechanisms of its secretion come into question. The classical secretory pathway of protein biosynthesis involves transfer from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane and depends upon the presence of a cleavable signal peptide [23]. Human XOR has no signal peptide [24,25] and is not known to be glycosylated, a consequence of the classical secretory pathway. However, increasing numbers of polypeptides with these characteristics, that are nevertheless secreted from both prokaryotic and eukaryotic cells, are being discovered [26] and it may well be that XOR is another such protein using a non-classical secretory pathway. In view of the relatively high affinity of XOR for heparin [18,19], it is interesting to note an earlier suggestion [27] that muscle L-14 lectin, exported by a non-classical pathway, would thus be separated from glycoconjugates, with which it interacted, until after its secretion. Similar considerations could apply to XOR, which may be expected to bind to cell surface glycosylaminoglycans following secretion. It is noteworthy that incubation of EA-hy-926 cells with heparin, followed by washing, failed to significantly diminish the intensity of staining (results not shown), suggesting that other glycosylaminoglycans may be involved.

Our results clearly show that XOR is not only present on the outer surface of cultured human endothelial and epithelial cells, but is asymmetrically distributed, in many cases appearing to be localised to surfaces apposed to those of closely neighbouring cells. This extracellular localisation and particularly its polarised nature strongly suggest a role for XOR in cell-cell interactions, possibly involving signalling via reactive oxygen species. We believe this to be an entirely novel concept worthy of detailed further investigations. Such investigations are, however, beyond the scope of the present study.

Acknowledgements: This work was supported in part by grants from the Arthritis and Rheumatism Council and Phytopharm Ltd. The

Biotechnology and Biological Sciences Research Council and the University of Bath are gratefully acknowledged for post-graduate research studentships (to S.P. and R.B. respectively). Mrs Joan Whish is gratefully acknowledged for excellent technical support.

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